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METABOLOMIC CHARACTERIZATION OF CANINE BEHAVIOURAL DISORDERS: FEARFULNESS AND HYPERACTIVITY/IMPULSIVITY

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Thank you for guiding me to the right path

Abstract

Psychiatric disorders in humans and their counterparts, behavioural disorders in dogs, are the major welfare problems in both species affecting the wellbeing of millions of people and dogs worldwide. Despite extensive research elucidating the pathobiological events leading to the onset of psychiatric/behavioural disorders, the exact etiology remains unattainable. Moreover, the complexity and multicausality of these mental disorders hampers research, addressing the need for more comprehensive approaches to provide insights into the molecular mechanisms of these disorders and to identify disorder-specific biomarkers.

The aim of this thesis was to pilot the use of a non-targeted metabolomics approach in canine behavioural research, and to provide novel molecular information concerning two specific behavioural disorders in pet dogs, fearfulness and hyperactivity/impulsivity.

Fearfulness is the most common behavioural disorder in dogs, characterized by excessive fear response when confronted with a threatening stimulus. During this thesis work, two separate non-targeted metabolomics characterization studies of canine fearfulness were conducted. The first study aimed to pilot the feasibility and potential of metabolomics technology in canine behavioural research, whereas the second metabolic characterization of canine fear was conducted with a larger sample size to optimize theoretical and analytical limitations observed in the first pilot study. The results showed clear differences in the blood metabolic profiles of fearful and non-fearful control dogs, including increased plasma glutamine abundance in fearful dogs. These alterations potentially originate from the systemic effects of chronic psychological stress.

Hyperactive/impulsive dogs manifest inappropriate levels of activity, impulsivity and inattention, corresponding to attention deficit hyperactive disorder (ADHD) in humans. The non-targeted plasma metabolomics showed lower levels of plasma phospholipids in addition to altered tryptophan metabolism in hyperactive and impulsive dogs. These changes may reflect disturbances in the gut microbiota composition in the affected dogs.

Collectively, this thesis has demonstrated the feasibility of metabolomics in canine behavioural research and provided novel molecular correlates and potential biomarkers for canine fearfulness and hyperactivity/impulsivity. Additionally, this study identifies changes which also had been reported in other species. This suggests that dogs could be used as a model to aid in gaining better understanding of human psychiatric disorders.

Tiivistelmä

Mielenterveyden häiriöt ihmisillä sekä koirien vastaavat tautitilat, käytöshäiriöt, ovat yleisimpiä elämänlaatua heikentäviä hyvinvointiongelmia molemmilla lajeilla. Laajamittaisista tutkimuksista huolimatta kyseisten tautitilojen etiologia on edelleen suurilta osin tuntematon. Sekä mielenterveyden häiriöt että käytöshäiriöt ovat taustaltaan monisyisiä sairauksia, mikä vaikeuttaa tutkimuksia. Sen vuoksi tarvitaan kokonaisvaltaisempia lähestymistapoja, jotka voivat tarjota lisätietoa tautitilojen molekulaarisista mekanismeista sekä tunnistaa tautikohtaisia merkkiaineita, biomarkkereita.

Tässä väitöskirjassa pilotoitiin kohdentamattoman metabolomiikan eli aineenvaihdunnan tutkimuksen käyttöä koirien käyttäytymistutkimuksessa. Tavoitteena oli tuottaa uutta molekyylitason tietoa kahdesta käytöshäiriöstä, arkuudesta sekä yliaktiivisuudesta ja impulsiivisuudesta.

Arkuus on yleisin koirilla esiintyvä käytöshäiriö, ja se ilmenee liiallisina pelkoreaktioina uhkaavan ärsykkeen, kuten toisen koiran tai vieraan ihmisen, läsnä ollessa. Tässä väitöskirjatyössä arkojen koirien aineenvaihduntaa tutkittiin kahdessa erillisessä tutkimuksessa. Ensimmäisen tutkimuksen tarkoitus oli testata metabolomiikan soveltuvuutta koirien käyttäytymistutkimuksessa, kun taas toisessa tutkimuksessa kehitettiin pilottitutkimuksessa havaittuja teoreettisia ja analyyttisiä puutteita suuremmassa aineistossa. Tulokset osoittivat, että arkojen ja ei-arkojen verrokkikoirien aineenvaihdunnassa oli selkeitä eroja, kuten kohonneet plasman glutamiinipitoisuudet aroilla koirilla. Nämä löydökset saattavat johtua arkojen koirien kärsimän kroonisen psykologisen stressin kokonaisvaltaisista seurauksista.

Yliaktiiviset ja impulsiiviset koirat kärsivät jatkuvasta ja sopimattomasta yliaktiivisuudesta, impulsiivisuudesta ja tarkkaamattomuudesta. Kyseinen tautitila vastaa ihmisillä esiintyvää tarkkaavuus- ja yliaktiivisuushäiriötä (ADHD). Metabolomiikka-analyysi paljasti madaltuneen fosfolipidien määrän sekä aminohappo tryptofaanin aineenvaihdunnan häiriöiden olevan yhteydessä koirien yliaktiiviseen ja impulsiiviseen käytökseen. Havaitut muutokset voivat heijastua häiriintyneestä suoliston mikrobitasapainosta.

Kaiken kaikkiaan tämä väitöskirjatyö on luonut pohjaa metabolomiikan laajemmalle käytölle koirien käyttäytymistutkimuksessa ja tarjonnut uutta molekyylitason tietoa koirien arkuudesta sekä hyperaktiivisuudesta ja impulsiivisuudesta. Lisäksi tutkimus on paljastanut molekyylitason yhtäläisyyksiä eri lajien välillä, mikä voi tulevaisuudessa hyödyttää eläinlääketieteen lisäksi myös ihmisen mielenterveyden häiriöiden tutkimusta.

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List of original publications

This thesis is based on the following publications:

I **Puurunen J**, Tiira K, Lehtonen M, Hanhineva K, Lohi H. Non-targeted metabolite profiling reveals changes in oxidative stress, tryptophan and lipid metabolisms in fearful dogs. *Behav Brain Funct.* 2016 Feb 12;12(1):7. DOI: 10.1186/s12993-016-0091-2

II **Puurunen J**, Tiira K, Vapalahti K, Lehtonen M, Hanhineva K, Lohi H. Fearful dogs have increased plasma glutamine and γ-glutamyl glutamine. *Sci Rep.* 2018 Oct 29;8(15976). DOI: 10.1038/s41598-018-34321-x

III **Puurunen J**, Sulkama S, Tiira K, Araujo C, Lehtonen M, Hanhineva K, Lohi H. A non-targeted metabolite profiling pilot study suggests that tryptophan and lipid metabolisms are linked with ADHD-like behaviours in dogs. *Behav Brain Funct.* 2016 Sep 29;12(1):27. DOI: 10.1186/s12993-016-0112-1

The publications are referred to in the text by their roman numerals.

Abbreviations

ADHD	Attention-deficit hyperactivity disorder
AUC	Area under the receiver operator characteristic curve
CCD	Canine compulsive disorder
CNS	Central nervous system
EDTA	Ethylenediaminetetraacetic acid
ENS	Enteric nervous system
ESI	Electrospray ionization
FDR	False discovery rate
GABA	γ-Aminobutyric acid
γ-Glu Gln	γ-glutamyl glutamine
HILIC	Hydrophilic interaction liquid chromatography
HPA	Hypothalamic-pituitary-adrenal
HPLC	High performance liquid chromatography
HRV	Heart rate variability
IAA	Indoleacetic acid
IDO	Indoleamine-2,3-dioxygenase
IPA	3-indolepropionic acid
KYNA	Kynurenic acid
LC	Liquid chromatography
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
OR	Odds ratio
PC	Phosphatidylcholine
PE	Phosphoethanolamine
PLS-DA	Partial least-squares discriminant analysis
PUFA	Polyunsaturated fatty acid
qTOF	Quadrupole time-of-flight
ROC	Receiver operator characteristic
ROS	Reactive oxygen species
RPLC	Reversed phase liquid chromatography
SDMA	Symmetric dimethylarginine
TDO	Tryptophan-2,3-dioxygenase
VIP	Variable importance on projection

1 Introduction

Dog, *Canis lupus familiaris*, is one of the most popular pets with worldwide population estimates of up to one billion dogs (Lord et al., 2013). Commonly, the dog is considered as man's best friend offering multiple emotional and health benefits for the owner (Hart, 1995; Mubanga et al., 2017). However, dogs may also cause major stress, embarrassment and even health hazards due to severe behavioural problems, such as fearfulness, separation anxiety or aggression (Gilchrist et al., 2008). These canine behavioural disorders are the leading causes of relinquishment and euthanasia of a dog (O'Neill et al., 2013; Salman et al., 2000). They are the most prevalent and major welfare problems in the dog population, which could have negative effects on the human community, too.

The human counterparts of canine behavioural disorders, psychiatric disorders, are also highly prevalent mental disorders with unfavourable effects to the individual's wellbeing (Asselmann et al., 2018; Dias et al., 2013; Overall, 2000; Steel et al., 2014). They are complex disorders that result from a mixture of multiple inheritable and environmental factors. Epidemiological and genetic studies have provided valuable information about the environmental and lifestyle influences and hereditary risk factors, respectively (Gratten et al., 2014; Meyer-Lindenberg and Tost, 2012). Nevertheless, the knowledge of how and to what extent these factors interact in the disorder pathology is still missing. Therefore, more comprehensive molecular technologies are necessary to uncover the exact underlying biological pathways of canine behavioural and human psychiatric disorders.

Metabolomics is an extensive analysis of all small-molecule metabolites in an organism (Beger et al., 2016; Dettmer et al., 2007). As metabolites are a result from the information flow from DNA to proteins, the metabolome is closest to phenotype. Thus, metabolomics is an ideal approach for investigation of complex diseases. It may provide novel information about disease mechanisms (*e.g.* genetic alterations or environmental exposures that are reflected as changes in the metabolome) and identify disease-specific biomarkers to facilitate diagnostics, treatment and disease management.

By piloting a non-targeted metabolomics approach for identification of behavioural disorder-specific metabolomic changes and putative biomarkers, this study has demonstrated the feasibility and potential of metabolomics in uncovering some molecular underpinnings of canine behavioural disorders with relevance to human psychiatric disorders. This study provides novel insights into the molecular mechanisms of two canine behavioural disorders, fearfulness and hyperactivity/impulsivity. A better understanding of these pathological states may not ultimately benefit just dogs, but could also establish models for biological aspects of human psychiatric disorders.

2 Review of the literature

The dog is believed to be the first domesticated animal species, originating from the gray wolf (*Canis lupus lupus*). The domestication is suggested to date back to time before the beginning of agriculture between 10,000 and 40,000 years ago, but the precise time and location remains controversial (Botigué et al., 2017; Frantz et al., 2016; Savolainen et al., 2002; Shannon et al., 2015; Thalmann et al., 2013; Vilà et al., 1997; Wang et al., 2016a). Moreover, it has been suggested that the dog would not have been domesticated just once but that multiple domestication events would have taken place at different times in multiple places (Frantz et al., 2016; Thalmann et al., 2013).

Although the domestication event dates back to ancient time, most of the over 400 current dog breeds are less than 200 years old (Lindblad-Toh et al., 2005; Vilà et al., 1997). These breeds encompass enormous variability in their morphology, physiology and behaviour as a consequence of recent strong artificial selection (Vaysse et al., 2011). Current selective practices are mainly driven by certain appearances appealing the dog breeders but for a long time dogs were selected mainly for their behavioural and working abilities like herding, hunting or guarding, to facilitate and benefit human life. As a result, certain behaviours were enriched in certain breeds, suggesting clear genetic components for different behavioural abilities (Lindsay, 2001). In addition, dogs have evolved advanced social cognition skills through the years of human and dog coexistence. Dogs are especially skillful at reading human social behaviours and they also easily seek help from the human (Miklósi et al., 2003). Similar communicative behaviours are not seen in wolves, suggesting that these abilities are not inherited from the wolves but instead result from the domestication process and close coexistence with humans during which dogs have adapted to live in close proximity to humans (Miklósi et al., 2003, 2004; Virányi et al., 2004).

2.1 The domestic dog as a natural animal model for human diseases

2.1.1 The research advantages of canine models

Population bottlenecks and intense artificial selection have resulted in narrowing genetic diversity of the dog, with subsequent enrichment of disease-causing mutations and risk alleles (Boyko 2011). This has led to the excess of inherited diseases in the dog. Many human diseases have close analogous or even homologous conditions in dogs where both the disease etiology and symptomatology are similar in both species (Hytönen and Lohi, 2016). As a prove of that, many disease-causing mutations in dogs are found in the same genes that are already associated with the corresponding human pathologies (Ahonen et al., 2013; Hytönen et al., 2016; Katz et al., 2005).

The high genetic similarity between human and dog (95%), and the unique breed-structure of the dog characterized by high heterogeneity between the breeds but extremely high homogeneity within the breeds facilitates especially genetic studies (Karlsson et al., 2007; Ostrander and Kruglyak, 2000; van Steenbeek et al., 2016). Due to the long-range linkage disequilibrium (LD) within the breeds, disease loci can be found in smaller study cohorts and with fewer markers than in humans (Hall and Wynne, 2012; Karlsson et al., 2007; Lindblad-Toh et al., 2005; Reich et al., 2001). An-example how findings in dogs can aid human medicine is narcolepsy. The identification of a mutation in hypocretin receptor 2 (*HCRTR2*) in Doberman pinchers as a causative for canine narcolepsy (Lin et al., 1999) offered novel understanding of the role of hypocretin system in human narcolepsies (Lin et al., 1999) with vast benefits in treatment development (Hoyer and Jacobson, 2013). Before this finding in dogs, hypocretin had not been considered as a potential candidate gene for narcolepsy in humans. Similar more recent examples exist in literature across disease groups (Hytönen and Lohi, 2016).

In addition to genetic similarity, as a large animal dog resemble humans also in respect of size and physiology. Furthermore, pet dogs share the same environment and similar lifestyles with humans as they consume similar food, are exposed to same pathogens and might even sleep in the same bed with their owners. They also resemble human in the terms of behaviour. These factors combined with a unique genetic architecture are important benefits in the study of complex diseases, such as allergies, cancers or psychiatric disorders where environmental factors are known to play a major role in the disease etiology. Therefore, dogs can offer a complementary and physiologically relevant animal model for many human diseases (Ostrander and Kruglyak, 2000). According to the OMIA database (Online Mendelian Inheritance in Animals, http://omia.org/home/), there are more than 400 suggested canine diseases or traits as models for human diseases. All this indicates that the domestic dog is a useful and important model for human

diseases and that research in canine companions can benefit both the dog and the human.

2.1.2 Dogs can help us to understand human psychiatric disorders

According to the *Diagnostic and Statistical Manual of Mental Disorders 5th edition* (DSM-5), psychiatric disorders are behavioural or psychological syndromes that are marked by clinically relevant disturbances in cognition, emotion regulation or behaviour, reflecting an underlying dysfunction of psychological, biological or developmental processes (American Psychiatric Association, 2013). They are prevalent disorders often characterized by incapacitating symptoms and high comorbidity which impair the patient's day-to-day performance and can result in adverse long-term outcomes, too (Asselmann et al., 2018; Steel et al., 2014).

Vulnerability to psychiatric disorders is highly heritable but genetically complex, *i.e.* many genes with only small effects influence on the development of the disorder (Gratten et al., 2014). Non-genetic factors, such as environmental exposures, play a major role and interact with the genetic factors, complicating the disorder etiology even more (Gershon et al., 2013; Toth, 2015). Thus, the genetic and molecular mechanisms behind mental disorders are still largely unknown, although massive efforts have been made recently (Anttila et al., 2018; Gratten et al., 2014). By utilizing large human study cohorts, novel model systems and more comprehensive study approaches, major breakthroughs are possible in the near future.

An innovative approach to untangle the biological background of psychiatric disorders is the use of animal models that spontaneously manifest conditions analogous or homologous to human diseases. Rodents are classical animal models in medical research, but they do not naturally manifest behavioural abnormalities similar to humans (Nestler and Hyman, 2010). There are also major physiological differences between rodents and humans. Thus, rodents are not the preferred animal models for human psychiatric disorders even though sometimes they might be the only possibility to gain knowledge to compare with.

Dogs have been aggressively bred for their appearance, use, temperament and behavioural abilities, such as herding, pointing and retrieving (Spady and Ostrander, 2008). Especially behavioural traits related to sociability, such as reduced fear and aggression, have been important in the selection process. This is demonstrated by a recent study suggesting that a positive selection of fear related variants on chr18 and chrX has been involved in the canid domestication (Zapata et al., 2016). The intense artificial selection has resulted in high behavioural variation among the dogs and in the enrichment of certain personality/behavioural traits in specific breeds (Duffy et al., 2008; Mehrkam and Wynne, 2014; Moon-Fanelli et al., 2011; Ogata et al., 2013; Scott and Fuller, 1965). This suggests genetic components also for canine behavioural

disorders. Even though candidate loci and genes have been demonstrated in canine behaviour, including social behaviour (Persson et al., 2016; vonHoldt et al., 2017) and canine compulsive disorder (Dodman et al., 2010; Tang et al., 2014), these findings are mainly suggestive.

Nevertheless, due to the unique genetic architecture, dogs are ideal subjects for behavioural research. Importantly, dogs spontaneously show behavioural abnormalities, such as fearfulness, impulsivity, and compulsions similar to humans (Overall, 2000). Moreover, they respond to same medication as human patients (Overall, 2001), suggesting that the biological mechanisms are shared between the species. Although dogs can never fully replace traditional animal models like rodents, they can aid research by gaining novel knowledge about disease mechanisms. Therefore, research resorting dog as a natural animal model for human psychiatric disorders has potential to benefit both the health of us and our best friend.

2.2 Canine behavioural disorders

2.2.1 Behavioural disorders are the major welfare problem in dogs

Behavioural problems are the major welfare issues and the leading causes for relinquishment or euthanasia of pet dogs (Miller et al., 1996; New et al., 2000; O'Neill et al., 2013; Patronek and Rowan, 1995; Salman et al., 2000). Sometimes canine behavioural problems may be due to management-related issues or underlying primary medical conditions, but often they are a result of abnormalities in the neural networks that regulate behaviour and cognition. These behavioural disorders are stable states of abnormal behaviour that interfere the performance and wellbeing of a dog. They are characterized by behavioural responses that are inappropriate in terms of frequency, duration and intensity or are aroused by harmless stimuli (van Rooy et al., 2014). Behavioural disorders encompass a variety of conditions in dogs, including fearfulness, noise sensitivity, separation anxiety, aggression, specific phobias (*e.g.* fear of heights or surfaces), canine compulsive disorder (CCD) and hyperactivity/impulsivity (**Fig. 1**).

Behavioural disorders comprise the major welfare problem in dogs due to several reasons. First, they are highly prevalent (Blackwell et al., 2013; Martínez et al., 2011; Tiira et al., 2016). Second, in extreme cases, the symptoms are severe causing major stress not only to the dog itself but also to its owner (Bennet and Rohlf, 2007; Meyer and Forkman, 2014; Serpell, 1996). Moreover, some behavioural problems like aggression may even cause severe public health threats (Gilchrist et al., 2008). Third, there is no consistent, reliable and objective way to identify and diagnose canine behavioural problems due to the high phenotypic heterogeneity of these disorders (van Rooy et al., 2014). As a result, behavioural problems might not be properly

identified and treated early enough, allowing them to become chronic, generalized and complicated by nature. Moreover, this hampers also research since study cohorts might not be properly constructed in the absence of objective phenotyping tools. Fourth, the medications used may not be efficient or can have severe side effects (Overall, 2001). Fifth, many behavioural problems tend to co-occur. For instance, fearful dogs manifest also noise sensitivity, separation anxiety and aggression (Tiira et al., 2016), complicating the identification, treatment and management of the problems. All these factors make canine behavioural disorders severe welfare problems, emphasizing the importance of addressing these issues.

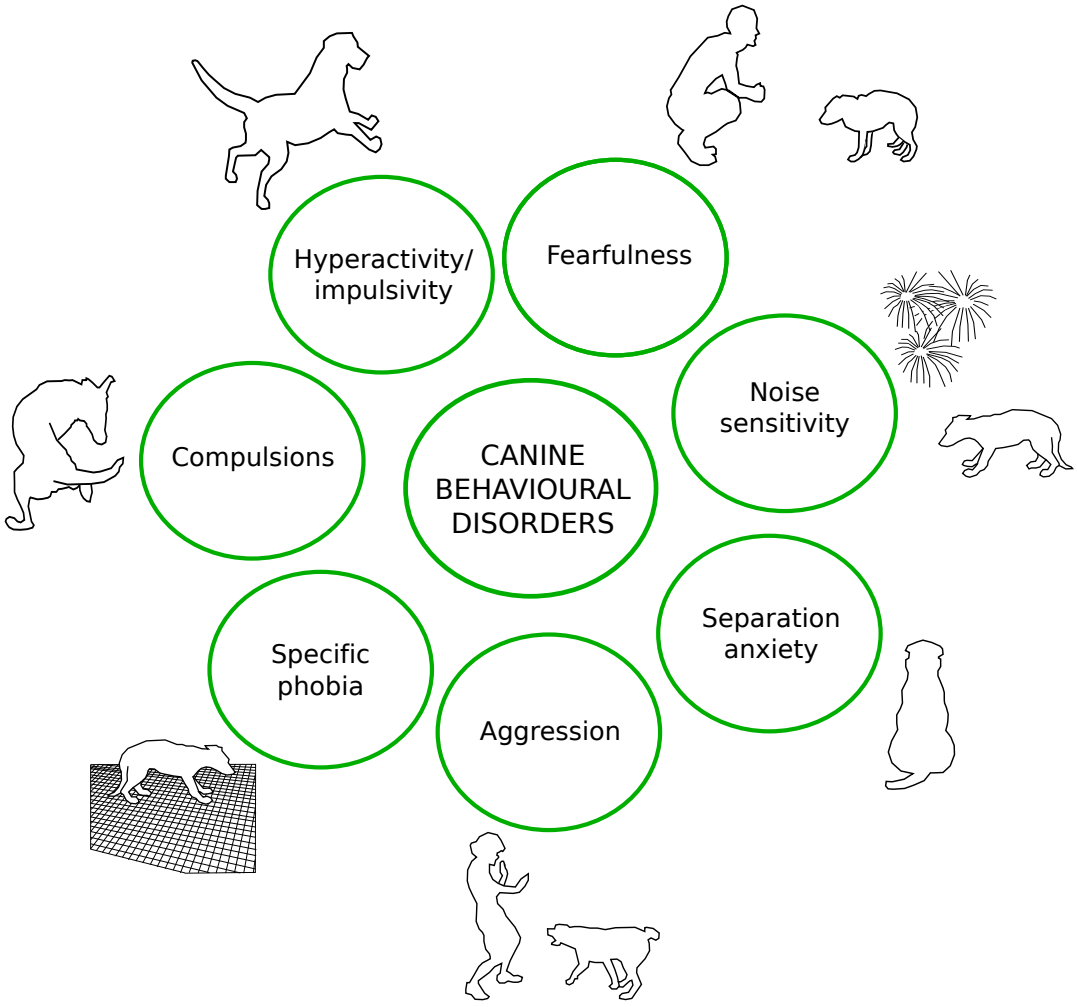

Figure 1. ***Canine behavioural disorders.*** Behavioural disorders, including fearfulness, noise sensitivity, separation anxiety, aggression, specific phobia, compulsions and hyperactivity/impulsivity, comprise a major welfare issue in the dog population.

The major reason for the lack of proper diagnostic and therapeutic options is the obscure and multifactorial etiology of canine behavioural disorders. Genes determine the foundation for individual's personality and behaviour, but non-genetic factors, such as environmental contributors shape them into the final outcome. Suggested candidate genes, relatively high heritability estimates, consistency of personality traits and breed-specificity indicate a major role for genetic predisposition (Duffy et al., 2008; Goddard and Beilharz, 1985; Moon-Fanelli et al., 2011; Ogata et al., 2013; van Rooy et al., 2014; Svartberg et al., 2005; Van Der Waaij et al., 2008). However, the impact of environmental factors, such as maternal environment, lack of early postnatal experiences, aversive learning and traumatic events is also large (Appleby et al., 2002; Barnard et al.; Foyer et al., 2013; Pluijmakers et al., 2010; Scott and Fuller, 1997; Tiira and Lohi, 2015). The problem lies in the determination of the complex interplay between these two factors, shaping the personality and behaviour of an individual into adverse, pathological direction. Moreover, the high individual variability in behaviour hinders the disclosure of the gene × environment interactions which could indicate why some are more vulnerable than others.

As this study focused on metabolic characterization of two specific canine behavioural disorders, fearfulness and hyperactivity/impulsivity, the characteristics of these behavioural abnormalities will be discussed in the following chapters.

2.2.2 Canine fearfulness

Fear is a fundamental emotional state, conserved among species and evoked by threatening stimuli (Adolphs, 2013; Anderson and Adolphs, 2014; Dias et al., 2013). As a transient feeling, fear is a normal emotion. It is often companied with acute stress reaction, and aimed to aid an individual to cope in threatening and challenging situations. From evolutionary perspective, fear is necessary for the fitness and survival of animals. However, if the fear is frequent or prolonged, the effects become negative leading to chronic stress with harmful consequences to the health and welfare of an affected individual (Schiavone et al., 2013). Persistent and repetitive fear can lead to anxiety, an emotional state of continuous distress and anticipation of potential danger (Hohoff, 2009). Anxiety is commonly characterized by generalized and long-lasting fear and distress as a response to perceived future danger or even in the absence of real threat. This distinguishes anxiety from fear which is an acute response to specific and known threat that resolves when the stimulus is no longer present. However, both of them are emotions with negative valence and harmful consequences if recurrent and continual.

Fearfulness is a personality trait on the shy-bold continuum, an axis of behavioural variation from shyness to boldness in animals (Kagan et al., 1988; Svartberg and Forkman, 2002). However, when recurrent, continual or

excessive, fearfulness becomes pathological and can manifest as a behavioural disorder with one of the highest prevalence rate of 26.2 % (Tiira et al., 2016). Moreover, the fear-related behavioural problems are common causes for relinquishment or euthanasia of a dog worldwide (Bamberger and Houpt, 2006; Miller et al., 1996; New et al., 2000; Salman et al., 2000).

Fear can manifest itself as many different visible behaviours in dogs. The most common clear expressions of fear include freezing/immobility, reduced posture, avoidance, escape and aggression (King et al., 2003; Scott and Fuller, 1997). Also other more subtle behaviours can be considered as indicators of fear, such as trembling, yawning, salivating, panting, paw-lifting, vocalizing, piloerection, autogrooming, repetitive behaviours (*e.g.* restless pacing), urination and defecation (King et al., 2003; Scott and Fuller, 1997). Based on the object of fear, canine fearfulness can be divided into social and non-social fearfulness. The social fear is directed either towards strangers or other dogs whereas the non-social fear is directed towards different objects, such as novel situations, surfaces or heights (specific phobia) and loud noises (noise sensitivity) (Svartberg, 2007). The nature and intensity of fear expression is determined by the stimuli: The type of stimuli (social vs non-social), the distance between dog and stimuli, the duration of the situation and previous experiences all have an impact to the final fear expression.

Fear and anxiety are characterized by specific physiological responses, mediated by several different pathways and circuits, that aim to facilitate the survival of an individual when encountering a threat. In the light of current knowledge, the core brain areas in fear processing encompass the amygdala, the thalamus, the sensory and association cortices, the medial prefrontal cortex (mPFC) and the hippocampus (Adolphs, 2013; Lang et al., 2000). The amygdala is the key structure, both receiving information from higher brain structures, such as the thalamus, and further passing it to lower structures, such as the hypothalamus, the bed nucleus of the stria terminalis (BNST) and the periaqueductal gray (PAG) (Davis, 1992; Steimer, 2002). These projections activate multiple physiological systems, such as sympathetic and parasympathetic nervous systems, and hypothalamic-pituitary-adrenal-cortical system ("HPA-axis"). These systems, in turn, are responsible for the specific physiological expressions of fear such as tachycardia, increased blood pressure, increased startle and vigilance, freezing and release of stress hormone cortisol, all of which can be seen as an altered behaviour (Davis, 1992) (**Fig. 2**). In addition, the thalamus and the amygdala project back to higher brain regions, such as sensory and association cortices, mPFC and hippocampus for more detailed processing of the fear (Calhoon and Tye, 2015). Moreover, many of the connections between different brain regions are reciprocal and certain, independent pathways are responsible for processing of different types of fear. This demonstrates the highly complicated nature of fear processing.

The neurotransmitters implicated in fear and anxiety include γ-aminobutyric acid (GABA), glutamate, dopamine, noradrenaline and

serotonin (Bukalo et al., 2014; Gross and Hen, 2004; Silveira Villarroel et al., 2018). In addition, a role for multiple neuropeptides, such as neuropeptide Y, has been demonstrated in fear and anxiety pathology (Silveira Villarroel et al., 2018).

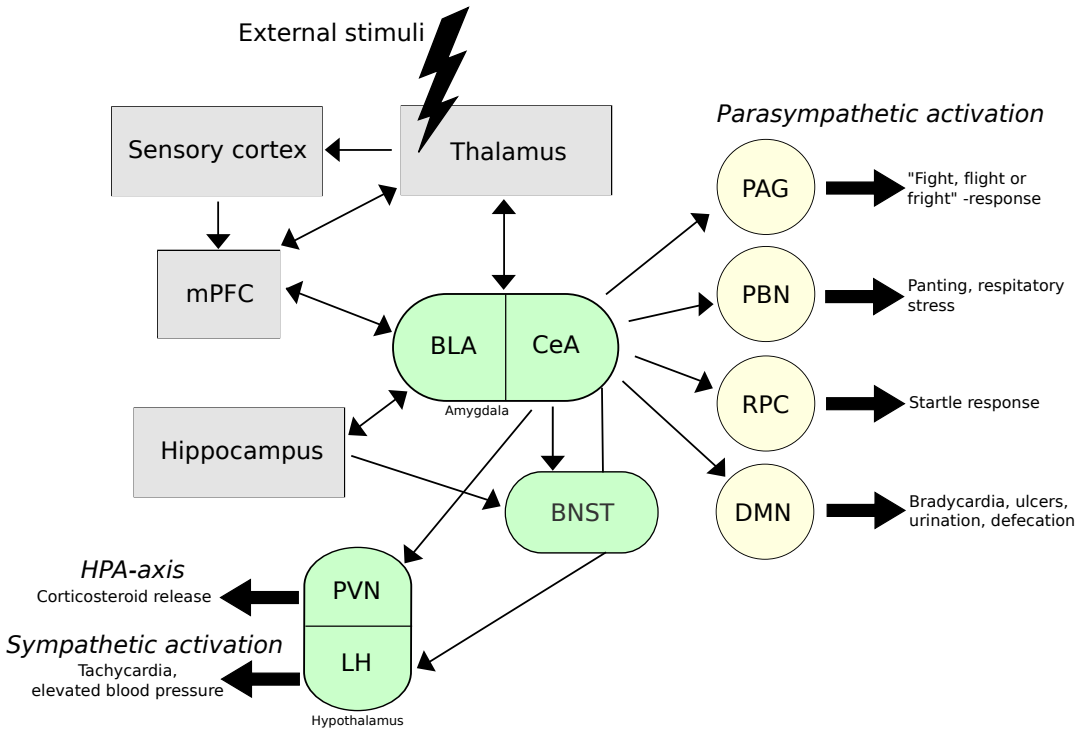

Figure 2. ***A simplified schematic presentation of the major brain structures involved in fear and anxiety processing.*** *The thalamus receives the external stimuli and passes the information to the cortical regions and the amygdala. The projections from the amygdala to lower brain structures are responsible for the physiological and behavioural manifestations of fear and anxiety. BLA, basolateral nucleus of amygdala; BNST, bed nucleus of the stria terminalis; CeA, central nucleus of amygdala; DMN, dorsal motor nucleus of the vagus nerve; mPFC, medial prefrontal cortex; LH, lateral hypothalamus; PAG, periaqueductal gray; PBN, parabrachial nucleus; PVN, paraventricular nucleus of the hypothalamus; RPC, caudal reticulopontine nucleus. The figure is adapted from Steimer 2002.*

Although the neurobiology of fear and anxiety as emotions is quite well established, the exact molecular mechanisms are still elusive. This results from the complex interplay between genetic and environmental factors in the

development of pathological fearfulness and anxiety. Heritability estimate of 0.49 has been suggested for canine fearfulness (Goddard and Beilharz, 1985), indicating strong genetic predisposition. However, only a few genomewide association (GWA) and candidate gene studies have been conducted in canine fear. In a Korean dog population, a role for *DRD4* polymorphism in fearfulness was suggested (Lee et al., 2008). A recent GWA study identified two promising loci, *GNAT3-CD36* on chr18 and *IGSF1* on chrX, associated with fear and aggression (Zapata et al., 2016). In addition, *DRD1*, IGF1 and PCDH9 have been suggested as candidate genes for trait boldness in dogs (Chase et al., 2009). Studies in other animals and humans have not been very successful, and only a minor amount of promising genetic risk factors have been identified, such as genes of serotonergic system and HPA-axis (Arnold et al., 2004; Hovatta and Barlow, 2008; Sokolowska and Hovatta, 2013).

Environmental factors have a large contribution to the development of personality and behaviour. In dogs, poor socialization in the puppyhood, aversive learning and traumatic events at any age are the most well established environmental factors associated with fearfulness (Appleby et al., 2002; Pluijmakers et al., 2010; Scott and Fuller, 1997; Serpell and Duffy, 2016; Tiira and Lohi, 2015). Canids, as well as many other animals, have a sensitive period for socialization in the early postnatal life. During this period, the nervous system is still developing and the brain is receptive for novel external stimuli, being an ideal time to habituate to the social world where living (Gazzano et al., 2008). However, lack of novel and positive experiences, or the presence of aversive events during this time period may permanently influence the neuronal development, reflected in the personality and behaviour of an individual. This early postnatal period is sensitive time especially for the development of systems controlling fear and stress responses, such as HPA-axis (Buttner, 2016; Daskalakis et al., 2013). Recently, other early-life factors, such as maternal care (Tiira and Lohi, 2015), litter (Foyer et al., 2013; Strandberg et al., 2005) and early weaning time (Pierantoni et al., 2011), have been identified to influence on the development of dog's behaviour. The effect of maternal care and its quality may be of special interest as it has been demonstrated to influence stress responses in several species through epigenetic mechanisms (Champagne, 2010).

2.2.3 Canine hyperactivity/impulsivity

Impulsivity is a stable behavioural trait observed across species (Dougherty et al., 2003; Riemer et al., 2014). It is marked by impaired self-control that manifests as inability to inhibit voluntary behavioural responses, leading to inappropriate actions. As a behavioural trait, impulsivity is an important part of a normal personality continuum. However, excessive, more frequent or prolonged manifestation of impulsivity is considered abnormal. Often, impulsivity is associated with aggression and violent behaviour, but it is also a

major component of several other conditions, including attention deficit hyperactivity disorder (ADHD) (Evenden, 1999; Vas et al., 2007; Wright et al., 2012).

According to the DSM-5, ADHD is a neurodevelopmental disorder characterized by inappropriate levels of motor hyperactivity, inattention and impulsivity (American Psychiatric Association, 2013). It is a childhood-onset disorder with prevalence rate of 3.4% in humans (Polanczyk et al., 2015). Individuals with ADHD have abnormalities in reward and attention processing, inhibitory control, and are also marked by emotional dysregulation (Gallo and Posner, 2016; Shaw et al., 2014). The symptoms, including restlessness, fidgeting, difficulties to focus or wait, and distractibility, are highly disabling and interfere the performance in normal day-to-day life (Biederman, 2005; Thapar and Cooper, 2016). Almost in half of the patients, ADHD can persist into adulthood. This may result in adverse long-term consequences, such as problems with social relationships, impaired academic functioning, substance abuse and underemployment (Gallo and Posner, 2016). ADHD also has several comorbidities, such as autism spectrum disorder, anxiety disorders and learning difficulties, further complicating the disorder phenotype.

Dogs also manifest abnormal levels of hyperactivity, impulsivity and inattention corresponding to human ADHD. Young dogs frequently show high levels of activity, impulsivity and inattention. However, especially working breeds, such as German Shepherd and Belgian Shepherd, continue to show inappropriate impulsivity also later in life since they were bred for high activity levels, enhanced alertness and vigilance to ensure their working dog properties (Lindsay, 2001). Moreover, there are large behavioural differences related to impulsivity even between the working and show lines within a breed (Fadel et al., 2016). The hyperactive/impulsive dogs are marked by high activity levels, excessive impulsiveness, lack of self-control and abnormally short attention spans, making them easily distracted and frustrated, restless and unable to concentrate long (**Table 1**) (Vas et al., 2007). In the most severe cases, the behaviours can lead to high levels of frustration and stress in the dog, and cause inconvenience and embarrassment also for the owner. Like human ADHD patients, hyperactive/impulsive dogs manifest reduced tolerance to the delay of reinforcement, indicating close similarity between species (Wright et al., 2012).

Table 1. ***The most common symptoms of canine hyperactivity/ impulsivity.***

Hyperactive/impulsive symptoms	Inattentive symptoms
Restlessness, fidgeting	Lack of attention
Loudness	Easily distracted
Frustration	Reacts hastily
Lack of self-control, impatience	

ADHD is a complex disorder marked by multifactorial etiology and high phenotypic heterogeneity (Thapar and Cooper, 2016; Thapar et al., 2013). Multiple neurobiological abnormalities, genetic risk variants and non-genetic risk factors have been implicated in abnormal levels of activity/impulsivity. However, the exact underlying molecular mechanisms are still poorly understood. This hinders the reliable identification of individuals with ADHD as well as the establishment of efficient treatment strategies.

The neurobiology of ADHD is characterized by alterations in mesocorticolimbic, frontoparietal and dorsal frontostriatal circuits that control reward, emotional and attention processes as well as inhibitory control (Biederman, 2005; Gallo and Posner, 2016). Structural abnormalities, such as differences in the volume of basal ganglia, a key brain structure in motor control and reward processing, altered cortical thickness in frontal and parietotemporal brain areas, and decreased white matter organization in aforementioned neural circuits have been implicated. Recent genetic, molecular, neuroimaging and pharmacological studies have shown impaired dopaminergic, noradrenergic and serotonergic neurotransmissions in ADHD pathology (Banerjee and Nandagopal, 2015; Faraone et al., 2005; Leo et al., 2003; Shim et al., 2016; Volkow et al., 2011).

ADHD runs in the families, as heritability estimates of 0.6-0.9 in humans suggest (Faraone et al., 2005; Gallo and Posner, 2016). Despite the high heritability, only a few genetic risk variants have been linked with ADHD across species, each variant having only minor effects. Most of the associated genes play a role either in the neurotransmitter systems implicated in ADHD, *i.e.* dopaminergic and serotonergic systems, or in synaptic transmission. In dogs, polymorphisms in genes encoding for dopamine D4 receptor (*DRD4*) (Hejjas et al., 2007a, 2007b, 2009; Wan et al., 2013), tyrosine hydroxylase (*TH*) (Kubinyi et al., 2012; Wan et al., 2013), dopamine transporter (*DAT*) (Hejjas et al., 2007b) and dopamine-*β*-hydroxylase (*DBH*) (Hejjas et al., 2007b) have been associated with hyperactive/impulsive behaviours. In addition, altered urine dopamine and serotonin metabolite levels have been linked with hyperactivity/impulsivity in dogs (Wright et al., 2012). This emphasizes the role of disturbed dopaminergic and serotonergic systems in

the pathology of hyperactivity/impulsivity. The identification of ADHD risk variants in the same genes in humans strengthens the assumption of similar molecular underpinnings in human ADHD and canine hyperactivity/impulsivity (Faraone et al., 2001, 2005; Gallo and Posner, 2016; Thapar et al., 2013).

Despite the high familiar risk of ADHD, non-genetic factors have large contributions too. Several pre- and postnatal factors, including low birthweight, prematurity, maternal stress and substance use during pregnancy, exposure to environmental toxins, psychological factors, such as extremely early social deprivation, family conflicts and early life stress, and dietary factors have been associated with ADHD in humans (Sciberras et al., 2017; Thapar and Cooper, 2016; Thapar et al., 2013). Also disturbances in the gut microbiota have been implicated to influence ADHD development via the gut-brain axis (Cenit et al., 2017). However, the causality of these environmental factors in the development of ADHD is still elusive. There is no studies investigating the environmental factors associated specifically with hyperactivity/impulsivity in dogs. However, similar factors, such as maternal stress, early weaning time, exposure to toxins or altered gut microbiota could be assumed to increase the risk for developing pathological hyperactivity/impulsivity also in dogs.

2.2.4 Biomarkers of canine behavioural disorders

One of the key factors in efficient management of canine behavioural disorders and corresponding human psychiatric disorders is reliable and early diagnosis of the disorders. This can be achieved by the utilization of biomarkers, molecules that can be objectively measured and evaluated as indicators or predictors of a disease (Sethi and Brietzke, 2015). Regrettably, there is a lack of valid and accurate biomarkers that could be easily and non-invasively measured to identify affected individuals and to differentiate pathologies.

In dogs, decreased 5HT-2A receptor binding in bilateral frontal, temporal and occipital cortical brain regions has been suggested as potential biomarker for anxiety (Vermeire et al., 2011). As the receptor binding in the brain is evaluated with neuroimaging by single photon emission tomography (SPECT), it does not offer practical and cost-effective biomarker for fluent everyday use. Instead, biomarkers of peripheral fluids, such as serum, plasma, urine or saliva, hold the greatest promise in early, cost-effective and minimally invasive or non-invasive diagnostics.

Elevated plasma dopamine and serotonin levels have been detected in anxious dogs (Riva et al., 2008), whereas lower urinary levels of serotonin metabolite 5-hydroxyindoleacetic acid (5-HIAA) and ratio between 5-HIAA and dopamine metabolite homovanillic acid (HVA) (5-HIAA/HVA) have been identified in hyperactive/impulsive dogs (Wright et al., 2012). These

peripheral measurements might have potential diagnostic value but larger validation cohorts are needed to verify their abilities as biomarkers.

2.3 Metabolomics in psychiatric and behavioural research

2.3.1 Introduction to metabolomics

Metabolome is the overall sum of all small molecules (< 1.5 kDa), called metabolites, found in a biological system (Beger et al., 2016; Dunn, 2008; Kaddurah-Daouk and Krishnan, 2009). Metabolites, such as amino acids, carbohydrates, lipids and nucleic acids, are both components and end products of basic biochemical pathways and processes. Thus, the metabolome reflects the functional cellular state of an individual at a given moment, influenced by both genetic and environmental factors and their interaction. Since metabolites are synthesized or otherwise transformed as a result from the information flow from DNA to RNA and further to enzymatically active proteins, metabolome is closest to phenotype in the ”omics cascade” (**Fig. 3**) (Dettmer et al., 2007). Therefore, the study of metabolism, metabolomics, has extensive potential to aid research.

Metabolomics aims to detect and measure hundreds to thousands of metabolites simultaneously with the ultimate goal to obtain a global snapshot of the metabolism of an organism (Dunn, 2008; Kaddurah-Daouk and Krishnan, 2009; Patti et al., 2012). As an omics-level approach, metabolomics can identify metabolite signatures related to health and disease states. Thus, metabolomics has great potential to identify disease-specific predictive or diagnostic biomarkers, predict therapeutic responses and uncover biological pathways and phenomena behind disease pathophysiology. Moreover, it is an ideal approach to investigate the effects of environmental and life style factors on complex diseases (Beger et al., 2016; Kaddurah-Daouk and Krishnan, 2009; Sethi and Brietzke, 2015).

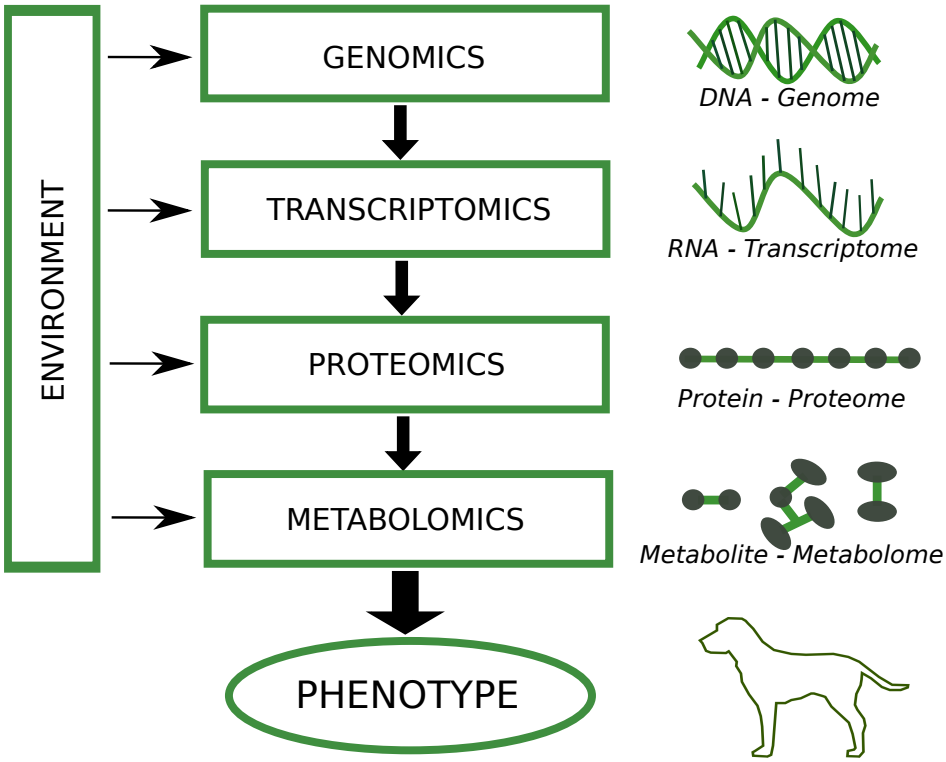

Figure 3. ***The information flow in the the "omics-cascade" from DNA to proteins and further to metabolites.*** *Environmental factors can interact with the genome, and those effects may be seen in the levels of transcriptome, proteome and metabolome. Metabolomics represents the endpoint of the cascade and thus is closer to phenotype providing real-time information about what has happened and is currently happening in an organism. Thus, metabolomics has the greatest potential to reveal changes related to health and disease states, but information from all omics levels are needed to obtain the most comprehensive biological understanding of complex systems. The figure is adapted from Dettmer et al. 2007.*

Metabolomics applications are typically divided to either targeted or non-targeted approaches (Dunn, 2008; Kaddurah-Daouk and Krishnan, 2009).

Targeted metabolomics refers to analysis of a particular and predetermined set of known metabolites which are of interest due to specific biochemical hypothesis or previous literature. In contrast, non-targeted metabolomics is an unbiased and hypothesis-free method aiming to measure as many metabolites as possible from a given sample with the chosen analytical instrumentation. Therefore, it offers a large-scale analysis of the current metabolic state. Liquid chromatography (LC) -based separation techniques for soluble analytes interfaced with mass spectrometry (MS) detector offers highly sensitive and accurate high-throughput approach for non-targeted metabolite profiling in health and disease (Dettmer et al., 2007; Dunn, 2008). Also other approaches, such as gas chromatography (GC) for volatile analytes followed by MS, or nuclear magnetic resonance (NMR) spectroscopy are utilized. However, LC-MS is usually the analytical platform of choice in global metabolomics studies. This is due to its low sample material requirement, the ability to reduce sample complexity and to detect the widest range of metabolites with different biochemical properties at variable concentrations (Dettmer et al., 2007; Dunn, 2008; Patti et al., 2012).

LC is a separation technique based on compound's interaction with the stationary phase in the LC column (Wilson, 2010). The affinity is dependent on the different chemical properties of molecules and its solubility in the given mobile phase composition, and thus analytes are eluted out of the column at specific time points, called retention times. Due to the wide physiochemical properties of different analytes, several chromatographic techniques are needed to maximize the metabolome coverage. The two main systems in use are reversed phase LC (RPLC) for more hydrophobic, non-polar metabolites (*e.g.* lipids) and hydrophilic interaction LC (HILIC) for hydrophilic, polar metabolites (*e.g.* amino acids, sugars) (Patti et al., 2012).

The compounds separated by LC are then analysed in a mass spectrometer. MS consists of an ion source, a mass analyzer and detector (Dunn, 2008). The role of ion source is to convert the electrically neutral analytes into charged molecular ions that can be further separated in a mass analyzer based on their mass-to-charge (m/z) values. In non-targeted metabolomics, electrospray ionization (ESI) is the commonly preferred choice of ion source because it is a soft ionization technique that produces intact molecular ions (Dettmer et al., 2007; Dunn, 2008). To detect a maximum number of metabolites, both negative and positive ionization modes are typically utilized. After ionization, the molecular ions are guided into the mass analyzer for separation, detection and conversion into accessible data, the mass spectrum (Dunn, 2008). Time-of-flight (TOF) instruments are the most utilized in non-targeted metabolomics due to its high sensitivity and mass accuracy (< 5 ppm). TOF instruments separate ions according to the flight time of an ion through the mass analyser: the smaller the m/z value (lower mass m and/or higher charge z) the faster the ion travels. In qTOF instruments the TOF analyser is interfaced with a quadrupole (q), an analyzer consisting of four parallel electrodes producing an electrostatic field. Ions passing thorough the rods are

then selected for detection by getting in resonance within a fast scanning radio frequency (RF) field. This hybrid qTOF technology allows to perform tandem mass spectrometry (MS/MS) experiments to gain further structural identification of compounds (Dettmer et al., 2007; Dunn, 2008). The quadrupole acts as a filter passing molecules based on their m/z values into a collision cell where the selected parent ions are fragmented into product/daughter ions, providing more detailed structural information of the analytes.

2.3.2 Metabolomics research on psychiatric and behavioural disorders

Several psychiatric disorders are characterized by biochemical abnormalities, which can be detected with the help of metabolomics (Kaddurah-Daouk and Krishnan, 2009; Sethi and Brietzke, 2015; Turck and Filiou, 2015). However, as metabolomics is still relatively novel research method, only few metabolomics studies of psychiatric and behavioural disorders in humans and rodents, and none in dogs, have been conducted in recent years. Some examples include anxiety (Filiou et al., 2011, 2014; Zhang et al., 2012), stress (Dulka et al., 2017), depression (Huang et al., 2017; Liu et al., 2016; Paige et al., 2007; Zhou et al., 2018), type D personality (Altmaier et al., 2013), aggression (Hagenbeek et al., 2016), schizophrenia (Fukushima et al., 2014; He et al., 2012; Kaddurah-Daouk et al., 2007) and autism (Wang et al., 2016b; West et al., 2014). Studies have provided some novel insights into these mental disorders, but so far the study cohorts have been relatively small and the results inconsistent.

2.3.3 Other study approaches

Efficient strategies in behavioural research include several methodologies, all of which can give valuable insights into different biological questions. Epidemiological studies have an important role in identifying environmental risk factors associated with behavioural disorders (Henderson, 2012), whereas genetic studies can gain information about the predisposing genetic alterations (Gratten et al., 2014). Additionally, transcriptomics and proteomics may help elucidating the molecular phenomena behind diseases by revealing disturbances in gene expression and protein function (Breen et al., 2016; Turck and Filiou, 2015). As the gene × environment interactions are known to be important in psychiatric and behavioural disorders, epigenetics have an essential role too (Mahgoub and Monteggia, 2013). As all these alterations, with additional effects from diet, environment, life style and gut microbiota, can be reflected in the metabolome of an individual, metabolomics represents the approach with the highest potential in psychiatric and

behavioural research (Beger et al., 2016; Kaddurah-Daouk and Krishnan, 2009; Turck and Filiou, 2015).

It is important to acknowledge that none of the study approaches alone is efficient enough when it comes to complex diseases. The highest potential lies in the combination of different omics -levels, *i.e.* systems biology: When all the different levels of omics approaches are applied together, the most comprehensive understanding of the pathophysiological factors leading to diseases can be obtained (Ala-Korpela et al., 2011). However, the challenge of systems biology is the successful integration and interpretation of the enormous amount of data from all different biological levels. Recent technical advances have facilitated data integration processes but more progress is still needed before large-scale systems biology studies can be conducted as a matter of routine. Moreover, very large study cohorts are needed to obtain significant and reliable results in metabolome-wide level.

In the context of psychiatric and behavioural disorders, the potential lies in the study approaches combining metabolomics with genomics (genome-wide association studies with metabolomics; mGWAS) (Ala-Korpela et al., 2011; Kastenmüller et al., 2015; Kettunen et al., 2012; Rhee et al., 2013), microbiome (gut microbiome-associated metabolomics) (Jansson et al., 2009; Yano et al., 2015) and pharmacology (pharmacometabolomics) (Kaddurah-Daouk et al., 2015). These comprehensive study approaches can gain information from multiple biological levels, offering powerful methods to untangle the molecular mechanisms of complex diseases.

3 Aims of the study

The primary goal of this PhD study was to characterize particular canine behavioural disorders, fearfulness and hyperactivity/impulsivity by a non-targeted metabolomics approach to reveal novel pathways associated with the disorder pathology and to identify potential disorder-specific biomarkers. Canine behavioural disorders are complex abnormalities caused by interplay between genetic and environmental factors. These effects were hypothesized to be reflected in the blood metabolite profiles, prompting us to pilot the use of a metabolomics approach in canine behavioural research.

The specific aims of this thesis were:

i. To characterize the potential of a non-targeted metabolomics approach in canine behavioural research (**Study I**)

ii. To investigate metabolic alterations in canine fearfulness (**Study II**)

iii. To identify metabolic changes related to ADHD-like behaviour in dogs (**Study III**)

4 Materials and methods

4.1 Ethics statement

All dogs were privately owned pet dogs which were enrolled in the studies with their owners' consent (**Study I-III**). The experiments were carried out under valid ethical licenses from the "Animal Ethics Committee at the State Provincial Office of Southern Finland" (**Study I-III**: ESAVI/6054/04.10.03/2012) and Royal Canin ethical board (**Study II-III**: 30052016).

4.2 Study cohorts

Professor Hannes Lohi has established a large canine DNA bank at the University of Helsinki, Finland, currently including over 70,000 DNA samples from more than 300 different breeds. In addition, the research group of Professor Lohi holds over a large behavioural data bank, based on owner-filled behavioural questionnaire and behavioural tests. With the resources provided by this behavioural data repository, Professor Lohi has established an ongoing canine behavioural disorder study cohort with the purpose to find environmental, genetic and metabolic determinants of canine behavioural traits with relevance to human psychiatric disorders.

The behavioural questionnaire includes both general questions concerning the dog's background, early life experiences and daily routines as well as more detailed questions of trait-specific behaviour, including fearfulness, noise sensitivity, aggression, separation anxiety, hyperactivity/impulsivity, stereotypic behaviour, specific phobias and canine cognitive dysfunction. The behavioural questionnaire has shown to have excellent reliability and validity, providing a reliable phenotyping tool for canine fear (Tiira and Lohi, 2014). In addition, the questionnaire part concerning dog's hyperactive/impulsive behaviour is based on previously validated questions on canine activity and impulsivity (Lit et al., 2010; Vas et al., 2007).

The dogs were selected for this study from a previously established canine anxiety cohort with the aim to find metabolic determinants of canine fearfulness and hyperactivity/impulsivity (**Study I-III**). To get questionnaire replies, the survey was advertised to Finnish breed clubs and dog owners via Facebook. Both owners of dogs with no sign of behavioural abnormalities as well as owners of dogs with behavioural problems were invited to fill-in the behavioural questionnaire. The dogs were selected for different studies as follows:

Study I: Based on questionnaire-derived behavioural variables (fearfulness (total), human fear frequency, human fear intensity and noise sensitivity), 10 fearful and 10 non-fearful Great Danes were recruited for the study. The human fear frequency variable corresponded to owner reported frequency of dog showing fear when meeting unfamiliar people, whereas the human fear intensity variable corresponded to the product of frequency of showing fearful behaviour multiplied by the sum of fearful behavioural reactions. The fearfulness variable corresponded to sum of frequencies of manifesting fearful reaction towards unfamiliar people, unfamiliar dogs and in novel situations. The noise sensitivity variable corresponded to the sum of frequencies of showing fearful reaction towards thunder, fireworks and gunshot. Fearful dogs have often (40-100% of occasions) showed fear at least towards strangers whereas non-fearful dogs have never indicated any sign of fear towards strangers, loud noises or in new situations/places according to their owners. The behaviour of the recruited dogs was confirmed by owner interviews and a short validated behavioural test was conducted to some of the dogs (Tiira and Lohi, 2014). The fearful and non-fearful dogs were age and sex matched.

Study II: Twenty fearful and 21 non-fearful Great Danes and German Shepherds were recruited for the study based on questionnaire-derived behavioural variables (human fear, situation fear and fear reaction). The frequency and intensity of fearful reactions towards strangers or in novel situations corresponded to human fear or situation fear variables, respectively. The fear reaction variable was a combination of these, describing the frequency and intensity of fearful reactions both in new situations and towards strangers. Non-fearful dogs have never showed any sign of fear in new situations either towards unfamiliar people or loud noises according to their owners, whereas fearful dogs have indicated fear towards unfamiliar people and/or novel situations often (40-100% of occasions). The behaviour of some of the dogs was verified by a short validated behavioural test (Tiira and Lohi, 2014), and owner interviews confirmed the behavioural patterns of the recruited dogs. The fearful and non-fearful dogs were breed, sex and age matched.

Study III: Twenty-two German Shepherds with varying hyperactive/impulsive behaviour were selected for the study. The dogs were selected based on three behavioural variables (inattention, impulsivity and total ADHD) describing the hyperactivity/impulsivity of the dog. The inattention and impulsivity variables were derived from factor analysis (PROC FACTOR analysis using the principal factor method with VARIMAX rotation) of the questionnaire questions concerning the impulsive and inattentive behaviour of the dog (SAS version 9.3., SAS Institute, Cary, NC, USA). The total ADHD variable was calculated as a mean of the answers to all questionnaire questions concerning the impulsive and inattentive behaviour.

The behavioural patterns of the recruited dogs were verified with owner interviews.

In **Studies II-III**, the recruited dogs consumed the same commercial dry food (Royal Canin Sensible) prior to sample collection for non-targeted metabolite profiling to control possible diet-effects. The summary of study cohorts is provided in **Table 2**, and more detailed description of the cohorts can be found in each individual study.

Table 2. ***Study cohorts.***

Study #	Studied behavioural disorder	Breed(s)	Behavioural variables	Sample material	Total number of dogs	Diet of dogs
I	Fearfulness	Great Dane	Fearfulness (total) Human_fear frequency Human_fear intensity Noise sensitivity	Whole EDTA blood	20	Not controlled
II	Fearfulness	Great Dane, German Shepherd	Human fear Situation fear Fear reaction	EDTA plasma	41	Royal Canin Sensible*
III	Hyperactivity/impulsivity	Great Dane	Total ADHD Hyperactivity Inanttention	EDTA plasma	22	Royal Canin Sensible*

* Diet changed for two weeks prior to sampling with one-week run-in period.

4.3 Non-targeted LC-MS metabolite profiling analyses

4.3.1 Sample collection

Ethylenediaminetetraacetic acid (EDTA) whole blood (**Study I**) or EDTA plasma (**Study II-III**) samples were collected from the recruited dogs for the non-targeted metabolite profiling analyses. The samples were drawn by cephalic venipuncture by a trained research personnel. In **Studies II** and **III**, the collected EDTA blood samples were immediately centrifuged to remove

cells from plasma. The samples were stored at -20°C prior to metabolomics analyses (**Study I-III**).

4.3.2 Sample preparation

Proteins were precipitated and metabolites extracted from EDTA whole blood (**Study I**) or plasma samples (**Study II-III**) prior to LC-MS analyses. In **Study I**, 400 µL of acetonitrile was added to 100 µL of whole blood sample, and in **Studies II** and **III** 300 µL of methanol was added to 100 µL of plasma sample. The samples were mixed in vortex at maximum speed 15 s, incubated on ice bath for 15 min and centrifuged at 16,000 × *g* for 10 min. The obtained supernatants were filtered with 0.2 µm Acrodisc® Syringe Filters with a PFTE membrane (PALL Corporation, Ann Arbor, MI) into HPLC vials (Agilent Technologies, Palo Alto, CA, USA). Quality control (QC) samples were prepared by combining aliquots of 10 µL (**Study I**) or 4 µL (**Study II-III**) from every supernatant.

4.3.3 HPLC-qTOF-MS analyses

LC-MS analyses and data preprocessing were carried out at the LC-MS Metabolomics Center at Biocenter Kuopio (University of Eastern Finland, Kuopio, Finland) (**Study I-III**). Extracted samples were analysed by UHPLC-qTOF-MS system (Agilent Technologies, Waldbronn, Karlsruhe, Germany) consisting of a 1290 HPLC system coupled with a Jetstream electrospray ionization (ESI) source and a 6540 UHD accurate-mass quadrupole time-of-flight (qTOF) spectrometer. To maximize metabolome coverage, two different chromatographic techniques (reversed phase liquid chromatography (RPLC) and hydrophilic interaction liquid chromatography (HILIC)) were utilized and data were acquired in both ionization polarities (positive (ESI+) and negative (ESI-)).

Two microliters of the sample were injected into Zorbax Eclipse XDB-C18 column (100 × 2.1 mm, 1.8 µm; Agilent Technologies, Palo Alto, CA, USA) and into Acquity UPLC BEH Amide column (100 × 2.1 mm, 1.7 µm; Waters Corporation, Milfrod, MA, USA) for RPLC and HILIC chromatographic separation, respectively. In RPLC, the column temperature was kept at 50°C and the mobile phases consisted of water (eluent A) and methanol (eluent B), both containing 0.1% (v/v) formic acid. The gradient profile was as follows: 0-10 min: 2 → 100 % B, 10-14.5 min: 100 % B, 14.5-14.51 min: 100 → 2 % B, 14.51-16.50 min: 2 % B at a flow rate of 0.4 mL/min. In HILIC, the column temperature was kept at 45°C and the mobile phases consisted of 50% (v/v) acetonitrile (eluent A) and 90% (v/v) acetonitrile (eluent B), both containing 20mM ammonium formate. The following gradient profile was employed: 0-

2.5 min: 100 % B, 2.5-10 min: 100 → 0 %, 10-10.01 min: 0 → 100 % B, 10.01-12.5 min: 100 % B at a flow rate of 0.6 ml/min.

The conditions of the MS ion source operated both in ESI+ and ESI- modes were as follows: drying gas temperature 325°C with a flow of 10 L/min, sheat gas temperature 350°C with a flow of 11 L/min, nebulizer pressure 45 psi, capillary voltage 100 V, and skimmer 45 V. For data acquisition, the mass range was set to 20-1,600 amu with an acquisition rate of 1.67 spectra/s. For automatic data dependent MS/MS analyses, the four most abundant ions were selected for fragmentation from every precursor scan cycle. After two product ion spectra, ions were excluded and released again after 0.25-min hold for fragmentation. The utilized collision energies were 10, 20 and 40 V. If the molecular ion of a compound was not included into automatic MS/MS analyses, targeted MS/MS fragmentation with collision energies 10 and 20 V was performed. For continuous mass axis calibration, the following ions from a reference mass solution were monitored throughout the runs: *m/z* 121.050873 and *m/z* 922.009798 in ESI+ and *m/z* 112.985587 and *m/z* 966.000725 in ESI-.

For quality assurance, QC samples were injected at the start, end and every 10th sample. The data acquisition was conducted by MassHunter Acquisition B.04.00 software (Agilent Technologies, Palo Alto, CA, USA).

4.3.4 Data collection and preprocessing

The metabolic features were extracted from the obtained LC-MS data with MassHunter Qualitative Analysis B.05.00 software (Agilent Technologies, Palo Alto, CA, USA), where the "Find by molecular feature" algorithm was utilized. The data was further output into Mass Profiler Professional (MPP) software (version 2.2., Agilent Technologies, Palo Alto, CA, USA) for compound alignment and data preprocessing. Background noise and low abundance metabolite features were excluded from further analysis according to their appearance and abundance in the samples. More detailed description of the preprocessing is provided in **Studies I-III**.

4.3.5 Annotation of metabolite features

Putative identification of detected metabolite features was performed based on accurate masses, retention times and fragmentation patterns acquired in the automatic data-dependent MS/MS acquisition. The MS/MS data and accurate masses were manually compared against online databases, including the Human Metabolome Database, the METLIN Metabolite Database, ChemSpider, SciFinder and KEGG, or earlier publications describing compound fragmentation patterns.

4.4 Statistical analyses

To find the metabolic features showing strongest associations with the canine behavioural disorders, several different statistical analyses were performed. Partial least-squares discriminant analysis (PLS-DA, Simca 13.0, Umetrics, Sweden) providing variable importance on projection (VIP) values for each metabolite was utilized to find the metabolite features having highest discriminating ability between fearful and non-fearful dogs (**Study I and II**) (Brereton and Lloyd, 2014; Sugimoto et al., 2012). To reveal differences in metabolic feature abundancies (signal intensities) between fearful and non-fearful dogs, fold change analyses (**Study I and II**) and Student's t-tests (**Study I**) were performed in MPP (version 2.2., Agilent Technologies, Palo Alto, CA, USA).

In **Study I**, further analysis and visualization of the metabolic alterations between fearful and non-fearful dogs was performed with *k*-Means clustering followed by hierarchical cluster analysis with heat map output (TM4 Microarray software) (Saeed et al., 2006).

Mann-Whitney U-test was used to determine the significant alterations in metabolic feature abundancies between fearful and non-fearful dogs in **Study II**. Furthermore, logistic regression analyses together with receiver operator characteristic (ROC) curve calculations were conducted to find the most discriminating metabolite features (SPSS 22.0.0.1, IBM, IBM Corp., Armonk, NY, USA; SAS version 9.3., SAS Institute, Cary, NC, USA). The discriminating abilities of the metabolites were evaluated based on odds ratios (OR) and area under the receiver operator characteristic curve (AUC) values derived from logistic regression and ROC curve analyses, respectively.

In **Study III,** Spearman correlation analysis was utilized to study associations between the metabolite feature abundances and behavioural variables (total ADHD, inattention and impulsivity variables). The possible effects of age, sex and fasting status on the observed associations were investigated by a partial correlation analysis (R project for Statistical Computing versions 3.0.1).

Benjamini-Hochberg false discovery rate (FDR) correction was used to control for multiple comparisons in **Studies I-III** (R project for Statistical Computing versions 3.0.1) (Benjamini and Hochberg, 1995).

5 Results

5.1 Canine fear is characterized by changes in phospholipid and amino acid metabolisms (Study I)

To assess the utilization and feasibility of LC-MS -based non-targeted metabolomics approach in canine behavioural research, a pilot study in a cohort of 10 fearful and 10 non-fearful age and sex matched Great Danes was conducted (**I/Table 1**). The age of fearful dogs varied from 1.1 to 9.3 years (mean 3.5 years) whereas the age of non-fearful dogs ranged from 1.1. to 8.5 years (mean 3.4 years). EDTA whole blood samples were collected for the metabolomics analysis. The diets of recruited dogs were retrospectively asked from the owners to consider the possible nutritional influences on observed metabolic profiles.

A total of 6,932 molecular features were detected in the LC-MS metabolomics analysis. Statistical filtering according to Student's t-test p-values $p < 0.05$, fold changes $\geq \pm 1.2$ (positive and negative fold changes indicate higher and lower abundance in fearful dogs, respectively) and VIP-values >1 resulted in a dataset of 239 features. Based on comparisons to databases or earlier publications (Berry and Murphy, 2004; Keller et al., 2013; Murphy and Axelsen, 2011; Ozawa et al., 2008; Xia and Jemal, 2009; Xu et al., 2009), 13 metabolites were identifiable and were reported together with five statistically significant but unknown metabolites (**I/Table 2**) (**Fig. 4**).

5.1.1 Altered blood phospholipid levels in fearful dogs

A majority of the differential metabolites between fearful and non-fearful dogs were phospholipids, including three phosphatidylcholines (PC) (PC(16:0/23:5), PC(18:0/19:1) and PC(18:0/20:4)), three LysoPCs (LysoPC(19:0), LysoPC(18:3) and unknown LysoPC with *m/z* 578.312), one Lyso-phosphatidylethanolamine (LysoPE) (LysoPE(20:5)) and one phosphatidylethanolamine plasmalogen (PE(P-18:1/20:4)) (**I/Table 2**). Fearful dogs had lower blood abundances of all other phospholipids except PE(P-18:1/20:4) which was increased. PC(16:0/23:5) showed the most remarkable difference between fearful and non-fearful dogs, being significantly decreased in fearful dogs (p_{raw}=0.002, p_{FDR}=0.023, fold change -2.06, VIP=2.24).

5.1.2 Changes in tryptophan metabolism and oxidative stress markers

Increased abundances of oxidative stress promoters hypoxanthine and indoxylsulfate (Chen et al., 2012; Muteliefu et al., 2009; Rodrigues et al., 2014) were detected in the blood of fearful dogs (hypoxanthine: p_{raw}=0.025,

p_{FDR}=0.123, fold change 1.87 and VIP=1.60; indoxylsulfate: p_{raw}=0.048, p_{FDR}=0.187, fold change 1.78 and VIP=1.85).

Indoxylsulfate is an indole-derived metabolite of tryptophan, an essential amino acid, and thus can reflect changes also in tryptophan metabolism (Dou et al., 2007). In addition, hypaphorine, an indole alkaloid and an N-methylated form of tryptophan was increased in fearful dogs (p_{raw}=0.049, p_{FDR}=0.172, fold change 2.17 and VIP=1.29). Finally, also tryptophan was identified as significantly decreased in fearful Great Danes when compared to non-fearful ones (p_{raw}=4.22E-04, p_{FDR}=0.087, fold change -1.58 and VIP=0.84). However, it was detected in RP but not in HILIC analysis which would be more reliable chromatographic method for amino acid detection. Thus, the significance this finding was questionable.

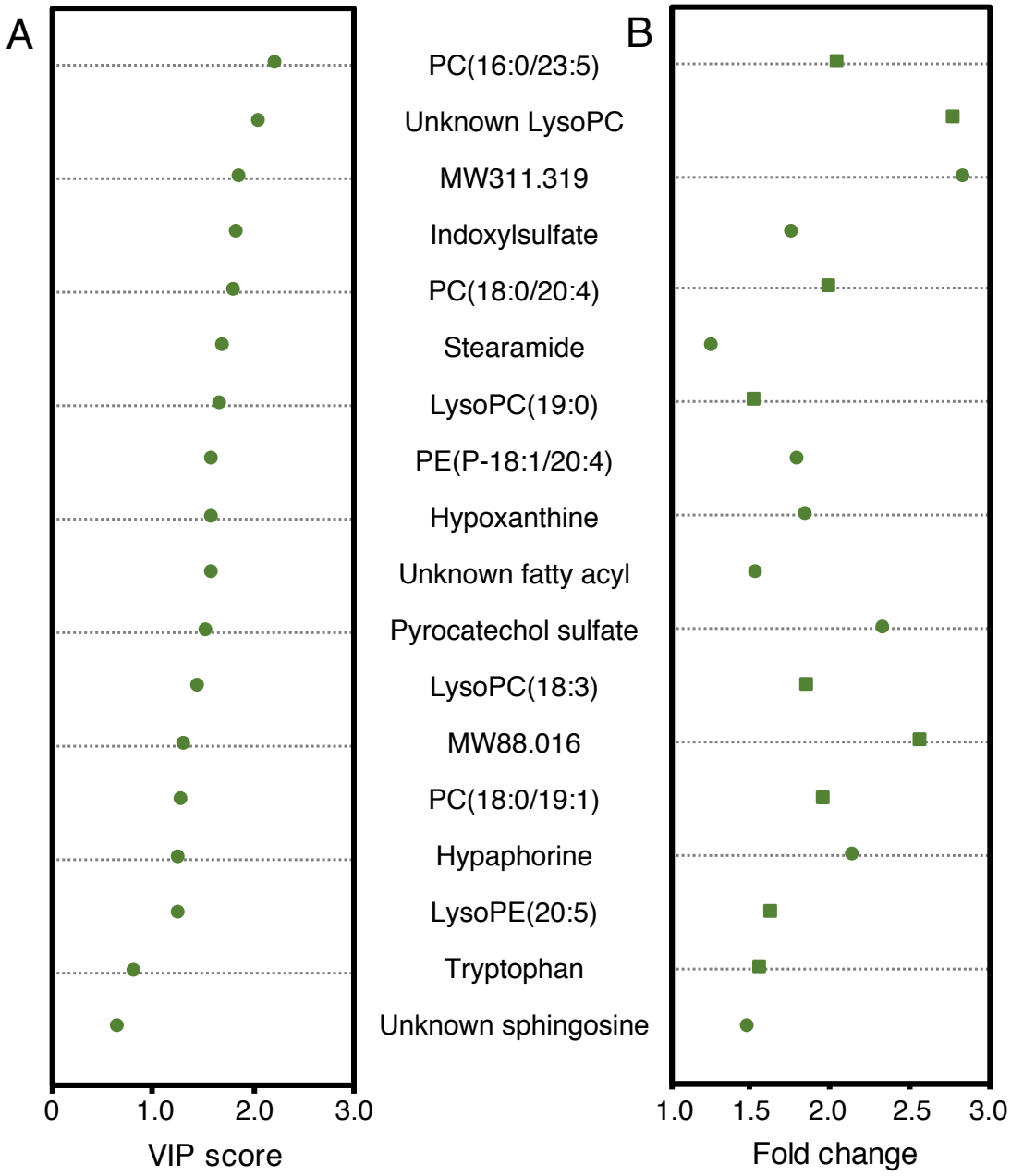

Figure 4. ***Distribution of VIP scores and fold changes among reported metabolites. A)*** *Distribution of VIP scores among reported metabolites.* ***B)*** *Distribution of fold changes among reported metabolites. Circles indicate positive fold changes (higher metabolite abundance in fearful dogs when compared to non-fearful dogs) whereas squares indicate negative fold changes (lower metabolite abundance in fearful dogs when compared to non-fearful dogs).*

5.2 Increased plasma glutamine is associated with canine fearfulness (Study II)

Twenty fearful and 21 non-fearful Great Danes and German Shepherds were selected for the breed, age and sex matched study cohort to investigate fear-related metabolic changes in canine plasma (**II/Table 1, S1 Table**). The age of fearful and non-fearful dogs varied from 1.5 to 8.7 years (mean 4.7 years, median 4.4 years) and 1.6 to 8.6 years (mean 4.6 years, median 4.3 years), respectively. Same commercial dry food was fed to all dogs, except one non-fearful German Shepherd, for two weeks before the blood sample collection. Part of the dogs were fasting 12 h before sampling.

The LC-MS -based metabolomics analysis resulted in a dataset of 6 718 molecular features which was further subjected to statistical filtering according to VIP-values >1, fold changes ≥ ±1.2 (positive and negative fold changes indicate higher and lower abundance in fearful dogs, respectively) and FDR corrected Mann-Whitney U p-values (p_{FDR}) <0.05 (**II/Fig. 1**). Altogether 41 metabolic features were included in the identification step but after manual filtering for poor chromatographic peak shapes or MS/MS fragmentation, five metabolites were identified and reported together with six unidentified metabolic features (**II/S2 Table**). The identification was based on comparisons to databases or earlier publications. These eleven metabolites were subjected to logistic regression analyses.

5.2.1 Elevated plasma glutamine and γ-Glu Gln levels associate with fearful behaviour

According to various statistical analyses, amino acid glutamine and γ-glutamyl dipeptide γ-glutamyl glutamine (γ-Glu Gln) were among the most significant discriminators between fearful and non-fearful dogs. PLS-DA analysis showed that both metabolites had high discriminating ability between the study groups (glutamine VIP=2.14 and γ-Glu Gln VIP=2.02) (**II/Fig. 2, S2 Table**). According to Mann-Whitney U-testing, the plasma abundances of glutamine and γ-Glu Gln differed significantly between fearful and non-fearful dogs (glutamine p_{FDR}=0.005 and γ-Glu Gln p_{FDR}=0.005), and also fold change analysis supports this finding (glutamine fold change 1.23 and γ-Glu Gln fold change 1.46) (**II/Fig. 2, S2 Table**). In addition, logistic regression analyses indicated that glutamine and γ-Glu Gln were the best discriminators between fearful and non-fearful dogs (glutamine OR=2.55 and p=0.004, γ-Glu Gln OR=3.10 and p=0.005) (**II/Table 2, Fig. 4**). They also had the highest diagnostic ability in ROC curve analysis (glutamine AUC=0.840 and γ-Glu Gln AUC=0.850) suggesting that glutamine and γ-Glu Gln could be potential biomarkers for fearfulness (**II/Table 3**). Breed, sex, age or fasting status had no significant effects on the phenotype (fearful/non-fearful) and they did not have significant interactions with the metabolites.

5.2.2 Renal biomarker SDMA shows breed-specific association with canine fearfulness

In addition to glutamine and γ-Glu Gln, the plasma abundance of symmetric dimethylarginine (SDMA), a marker of renal function, was highly differential between fearful and non-fearful dogs but in a breed-specific manner. In univariate analyses where breed was not included as a confounding factor, a clear difference in SDMA levels was observed between fearful and non-fearful dogs (VIP=2.16, p_{FDR}=0.032, fold change 1.98) (**II/Fig. 2, S2 Table**). However, the logistic regression analysis revealed a significant interaction between SDMA and breed, indicating that SDMA was significantly associated with fearfulness in Great Danes (OR=1.83, p=0.004) but not in German Shepherds (OR=0.98, p=0.902) (**II/Table 2, Fig. 4**).

To find the best combination of metabolites as predictors of fearfulness, multiple logistic regression analysis was conducted. Glutamine and SDMA together were identified as best discriminators between fearful and non-fearful dogs although the multiple logistic regression analysis was hindered by high multicollinearity between the individual metabolites. The breed-specificity was visible also in this model - higher plasma SDMA abundance was significantly associated with fearfulness in Great Danes (OR=2.23), but there was a minor, non-significant negative association between fear and SDMA abundance in German Shepherds (OR=0.80) (**II/Table 4, Fig. 5**). The association between higher plasma glutamine and fear was highly significant (OR=3.15).

5.3 Tryptophan and lipid metabolites are linked with ADHD-like behaviours in dogs (Study III)

A cohort of 22 German Shepherds with ADHD-like behaviours varying from mild to high was recruited to study. The dogs' ADHD-like behaviours were measured and described by continuous behavioural variables (total ADHD, inattention and impulsivity variables) that varied from 1.0 (mildest) to 3.8 (highest) (the higher the behavioural score, the more the dog had ADHD-like behaviour) (**III/S2 Table**). The age of the dogs ranged from 1.3 to 7.5 years (mean 5.1 years, median 5.4 years). All dogs consumed same commercial dry food two weeks prior blood sampling to control the possible dietary effects, and majority of the dogs were fasting 12 h before the plasma collection.

The LC-MS analysis detected altogether 7 058 molecular features from which 649 correlated either with one or more behavioural variables (p_{raw} <0.05). Twenty-two metabolites were putatively identified based on comparisons to databases or earlier publications (**III/Table 1**). Previously described characteristic fragmentation patterns of lipid compounds were utilized to identify PCs, LysoPCs and LysoPEs (Murphy and Axelsen, 2011; Xia and Jemal, 2009; Xu et al., 2009). Also five unknown metabolites were reported. Majority of the reported metabolites (20 out of 27) were associated

with all three ADHD-like behavioural variables (**III/Fig. 1, Table 2**). However, phenylalanine was associated only with the impulsivity variable, and sn-1 LysoPC(14:0), sn-1 LysoPC(18:1), unknown PC with *m/z* 796.516 and indoleacetic acid (IAA) only with the inattention variable.

5.3.1 Tryptophan metabolites associate with canine ADHD-like behaviour

Three tryptophan metabolites identified as 3-indolepropionic acid (IPA), kynurenic acid (KYNA) and indoleacetic acid (IAA), correlated with ADHD-like behavioural scores (**III/Fig. 1, Table 2**). The plasma IPA abundance was negatively associated with all three behavioural variables and had the strongest association with inattentive behaviour (Total: r_s=-0.565, p_{raw}=0.006, p_{FDR}=0.381; Inattention: r_s=-0.618, p_{raw}=0.002, p_{FDR}=0.241; Impulsivity: r_s=-0.452, p_{raw}=0.035 p_{FDR}=0.560). The association between IPA and impulsivity variable was nominally significant when age, sex and fasting status were controlled in the analysis (original p=0.035, adjusted p=0.058) (**III/Table 3**).

In contrast to IPA, the plasma KYNA abundance had positive associations with ADHD-like behaviours (Total: r_s=0.511, p_{raw}=0.015, p_{FDR}=0.426; Inattention: r_s=0.505, p_{raw}=0.017, p_{FDR}=0.392; Impulsivity: r_s=0.498, p_{raw}=0.018 p_{FDR}=0.447) (**III/Fig. 1, Table 2**). IAA was associated only with inattentive behaviour, showing negative correlation with the inattention variable (r_s=-0.474, p_{raw}=0.030, p_{FDR}=0.433), but the association was no longer significant after controlling for age, sex and fasting effects (original p=0.030, adjusted p=0.062) (**III/Fig. 1, Table 2, Table 3**).

5.3.2 The intensity of ADHD-like behaviour is negatively correlated with plasma phospholipid levels

More intense ADHD-like behaviour was characterized by lower plasma lipid abundances, since phospholipids were the major compound class associated with ADHD-like behaviours in the study cohort (**Fig. 5**). In total, five PCs (PC(18:3/18:2), PC(20:5/18:3), PC(20:4/14:0), PC(18:2/16:1) and PC(15:0/18:2)), five LysoPCs (sn-1 LysoPC(18:3), sn-1 LysoPC(14:0), sn-1 LysoPC(15:0), sn-1 LysoPC(17:0) and sn-1 LysoPC(20:3)), four LysoPEs (sn-1 LysoPE(18:2), sn-1 LysoPE(20:5), sn-1 LysoPE(18:1) and sn-1 LysoPE(18:0)), and one unknown PC with *m/z* 796.516 had negative correlations with one or more ADHD-like behavioural variables (**III/Fig. 1, Table 2**). The associations between sn-1 LysoPC(18:3) and all three behavioural variables (Total: r_s=-0.769, p_{raw}=2.93E-05, p_{FDR}=0.016; Inattention: r_s=-0.740, p_{raw}=8.26E-05, p_{FDR}=0.030; Impulsivity: r_s=-0.765, p_{raw}=3.32E-05 p_{FDR}=0.036), PC(18:3/18:2) and total and inattention variables (Total: r_s=-0.804, p_{raw}=1.91E-05, p_{FDR}=0.0.16; Inattention: r_s=-0.798, p_{raw}=2.53E-05, p_{FDR}=0.0.019), and sn-1 LysoPE(18:2) and inattention variable (Inattention:

r_s=-0.697, p_{raw}=3.1E.04, p_{FDR}=0.044) remained significant after FDR correction for multiple comparisons. In contrast, the plasma abundances of two fatty acids, arachidonic acid (C20:4) and C18:1, were positively correlated with the ADHD-like behavioural variables.

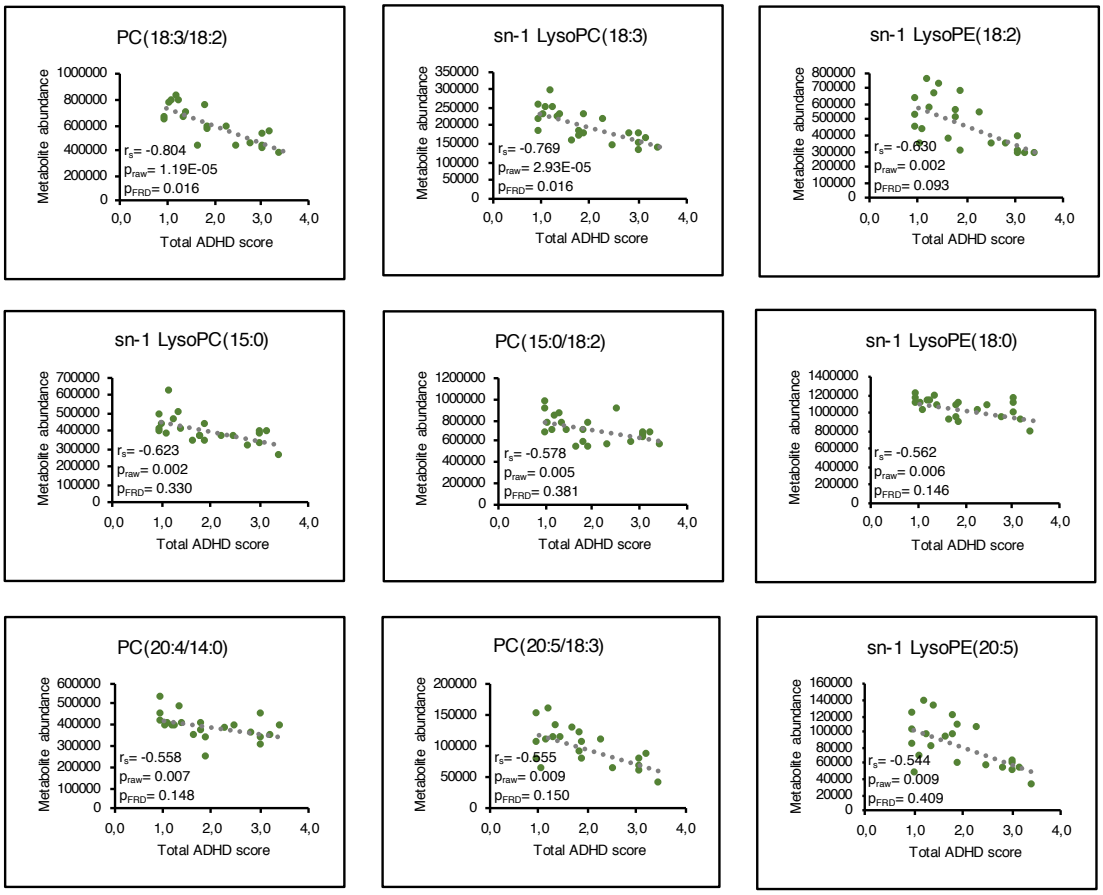

Figure 5. ***Correlation scatter panel of phospholipids that significantly (p_{raw} < 0.01) correlated with total ADHD behavioural variable.*** *Spearman correlation coefficients (r_s), raw p-values (p_{raw}) and FDR corrected p-values (p_{FDR}) are reported.*

6 Discussion

This study focused on pioneering the utilization of a non-targeted metabolomics analysis in canine behavioural research, with specific emphasis on fearfulness and hyperactivity/impulsivity. Significant and biologically relevant trait-specific metabolic alterations were identified. This demonstrates the feasibility and potential of metabolomics approach to uncover molecular phenomena of and to identify biomarkers for canine behavioural disorders.

In this study, several novel metabolites, such as SDMA and IPA, were associated with canine fearfulness and hyperactivity/impulsivity, respectively. Metabolic alterations previously linked to corresponding disease states in humans or rodent models were also identified, including decreased abundance of circulating lipids, increased levels of oxidative stress markers and gut bacteria derived metabolic changes in dogs with behavioural abnormalities. This highlights the biological similarities in the etiology of these disorders across species, and strengthens the role of the domestic dog as a natural animal model for human psychiatric disorders.

In this thesis, two different studies of metabolomics of canine fearfulness were conducted with two different study cohorts (**Study I-II**), but the results were not replicated. This discrepancy can be explained by several different factors, including different sample material (EDTA whole blood vs. plasma), differences in the analytical conditions (acetonitrile vs. methanol used for protein precipitation), nutritional differences and small sample sizes.

Despite the relatively small sample sizes in this study, significant metabolic differences were detected in each trait, owing to the careful and thorough selection of the study cohorts. However, this study also underlines the common challenges associated with non-targeted metabolomics studies, including deficient metabolite identification and multiple comparisons problem, arising from the large amount of data that is obtained. In addition, the causality of the findings remains unsolved since metabolomics analyses alone do not reveal if the observed metabolic alterations are the reason for or the result of the studied problem. Nevertheless, the results demonstrate that dogs with behavioural abnormalities are characterized by specific metabolic profiles, providing us novel insights into the etiology of canine behavioural disorders.

6.1 Metabolic alterations observed in fearful dogs may result from systemic effects of chronic psychological and oxidative stress

This study investigated metabolic alterations between fearful and non-fearful dogs in two different study cohorts, one consisting of 20 Great Danes (**Study I**) and the other one of 41 Great Danes and German Shepherds (**Study II**). Increased abundances of metabolites related chronic stress (glutamine and SDMA) and oxidative stress (hypoxanthine, indoxylsulfate, PE(P-18:1/20:4) and γ-Glu Gln) were identified in fearful dogs. The associations between these metabolites and fearfulness are novel and suggest that chronic psychological stress and subsequent oxidative stress may play a role in canine fear.

Many, especially anxiety-related, mental health issues are commonly associated with high levels of chronic stress when the surrounding environment is too demanding to cope with (Schiavone et al., 2013). Also highly fearful dogs that manifest excessive and frequent fear can suffer from prolonged psychological stress as they might be in continuous state of alertness and anxiety (Dreschel and Granger, 2005; Hydbring-Sandberg et al., 2004). Moreover, fearful dogs may have perturbations in the stress-response systems, making them more prone to the everyday stress. Although transient stress has positive valence by facilitating an individual to cope in challenging situations, chronic stress has several negative health effects not only on the mental health but also on cardiovascular, immune and neuroendocrine systems. Prolonged stress causes chronic activation of sympathetic nervous system and HPA-axis, resulting in increased catecholamine and cortisol release and elevated cardiac output and hypertension, for instance. Moreover, sustained stress may permanently alter the HPA-axis function, predisposing individuals to more severe psychiatric problems (Colaianna et al., 2013; Schiavone et al., 2013).

Glutamine is the most abundant amino acid in mammals and essential for several vital biological reactions (Curi et al., 2005; Matés et al., 2002; Schousboe, 2018; Weiner and Verlander, 2013). Most of the circulating glutamine is originated from the skeletal muscle whereas intestine, immune system, kidney and liver metabolize most of the free glutamine (Brosnan, 2003; van der Hulst et al., 1996). In addition, a part of glutamine is utilized for neurotransmitter synthesis in the CNS, including production of primary excitatory neurotransmitter glutamate via glutamate-glutamine cycle (Bergink, 2004; Cooper, 2001; Schousboe, 2018). Disturbed function of the glutamate-glutamine cycle has been linked with multiple psychiatric conditions (Cortese and Phan, 2005; Filiou et al., 2011, 2014; He et al., 2012; Jelen et al., 2018), highlighting the significance of adequate glutamine levels in several health and disease states.

The diet, the rates of *de novo* amino acid synthesis and protein degradation (Cynober, 2002) together with glutamine clearance from the brain (Hawkins et al., 2006; Xiang et al., 2003) determine circulating glutamine levels. Also cortisol, “a stress hormone” synthetized in the adrenal glands as a consequence

of the HPA-axis activation, may affect glutamine levels. As cortisol regulates breakdown of proteins as well as *de novo* synthesis of amino acids (Cynober, 2002), sustained elevation in cortisol secretion due to prolonged stress may lead to increased excretion glutamine from tissues to circulation.

In this study, canine fearfulness was associated with elevated plasma abundance of SDMA in a breed-specific manner, *i.e.* fearful Great Danes but not fearful German Shepherds had increased plasma SDMA. SDMA is a non-proteinogenic amino acid produced by post-translational modifications and released via proteolysis into circulation from where it is eliminated largely by the kidneys (Tain and Hsu, 2017). It is used as a marker of renal function in dogs as increased circulating SDMA levels have been linked to acute kidney injury and chronic kidney disease (Dahlem et al., 2017; Hall et al., 2016; Nabity et al., 2015). Since plasma SDMA concentration is solely affected by renal factors (Nabity et al., 2015), these results suggest that the renal function of fearful Great Danes may be compromised. This might be explained by chronic psychological stress causing sustained hypertension (Höglund et al., 2012; Wilhelmj et al., 1952) that leads to reduced glomerular filtration capacity of the kidneys. This often results in renal dysfunction and subsequent accumulation of SDMA in the circulation (Bartges et al., 1996; Rutledge and Hogan, 2002; Saran et al., 2016; Sparrenberger et al., 2009). Reduced heart rate variability (HRV) has been associated with anxiety in both dogs (Wormald et al., 2017) and humans (Bajkó et al., 2012; Gorman and Sloan, 2000). Our findings presumably originating from adverse effects of hypertension in fearful dogs are in line with these studies since HRV inversely correlates with blood pressure, indicating impaired function of autonomic nervous system.

However, the breed-specificity of SDMA elevation raises some questions. Individuals differ in their stress-sensitivity and stress-reactivity, and genetic and epigenetic alterations in the major stress-response pathways, such as the HPA-axis and the sympathetic nervous system, have been implicated to mediate these differences. As a result, some are more prone to everyday stressors than others (Chung et al., 2009; Daskalakis et al., 2013; Imumorin et al., 2005; Li-Tempel et al., 2016; Maccari et al., 2014; Picard et al., 2015). Since Great Danes and German Shepherds differ greatly in their looks, size and uses, they presumably also differ in terms of physiology and genetics. This might be reflected in differential adaptive stress responses and subsequent metabolic alterations, as our finding of elevated SDMA plasma abundance in fearful Great Danes but not in German Shepherds suggests.

Fearful dogs showed increased blood abundance of hypoxanthine and indoxylsulfate, uremic toxins promoting ROS production (Chen et al., 2012; Dou et al., 2007; Rodrigues et al., 2014). This suggests that they might suffer from oxidative stress, a state of imbalance between the production of ROS and antioxidant defense. Plasmalogen PE(P-18:1/20:4) acts as an antioxidant (Engelmann et al., 1994; Lessig and Fuchs, 2010), and decreased plasmalogen levels have been linked with increased oxidative stress (Brosche et al., 2013; Colas et al., 2011). Elevated PE(P-18:1/20:4) abundance in fearful dogs might

represent a secondary response to oxidative stress. Besides those markers, an indirect indication of oxidative stress was revealed as fearful dogs showed higher plasma abundance of γ-Glu Gln. γ-glutamyl dipeptides are produced as a by-product in breakdown of antioxidant glutathione, a reaction that is enhanced by oxidative stress (Liu et al., 2014). This further consolidates the finding that oxidative stress may play a role in canine anxiety as metabolites indicating high oxidative stress levels were identified in two separate study cohorts.

Indoxylsulfate is produced from dietary tryptophan by enteric bacteria (Keszthelyi et al., 2009; Meyer and Hostetter, 2012), and increased plasma indoxylsulfate has been suggested to indicate early-phase renal failure with changes in the gut microbiota in humans (Barrios et al., 2015). Moreover, also elevated hypoxanthine levels have been linked with kidney dysfunction (Chen et al., 2012; Rodrigues et al., 2014). The reason for these associations most probably lies in the high susceptibility of kidneys for the adverse effects of oxidative stress (Ozbek, 2012). As we detected also increased level of renal dysfunction biomarker SDMA in fearful Great Danes, together these data suggest that the renal function of fearful dogs might be compromised due to the adverse systemic physiological effects of sustained psychological and oxidative stress.

Chronic psychological stress has been demonstrated to predispose to psychiatric disorders and oxidative stress is suggested to mediate this effect (Aschbacher et al., 2013; Dulka et al., 2017; Nedic Erjavec et al., 2018; Schiavone et al., 2013). However, the mechanisms behind this association are not clear. One proposed pathway is the HPA-axis which is suggested to be upregulated not only by the chronic stress but also by the oxidative stress, the latter one being stimulated by the sustained stress (Asaba et al., 2004; Colaianna et al., 2013). As the circulating levels of all these discussed metabolites can be affected by the function of HPA-axis, it can be speculated whether altered HPA-axis function could play a role also in canine fearfulness.

Taken together, our finding of elevated abundances of glutamine, SDMA, hypoxanthine, indoxylsulfate, PE(P-18:1/20:4) and γ-Glu Gln in fearful dogs may reflect the adverse physiological effects of chronic psychological stress (**Fig. 6**). As sustained stress and increased oxidative stress has been frequently associated with psychiatric disorders in humans and in animal models, our results are in line with these findings. This highlights the similar etiology of anxieties among species and emphasizes the role of the domestic dog as a natural animal model for human anxiety disorders. Nevertheless, further studies are required to confirm and clarify the role of these metabolites in canine fear.

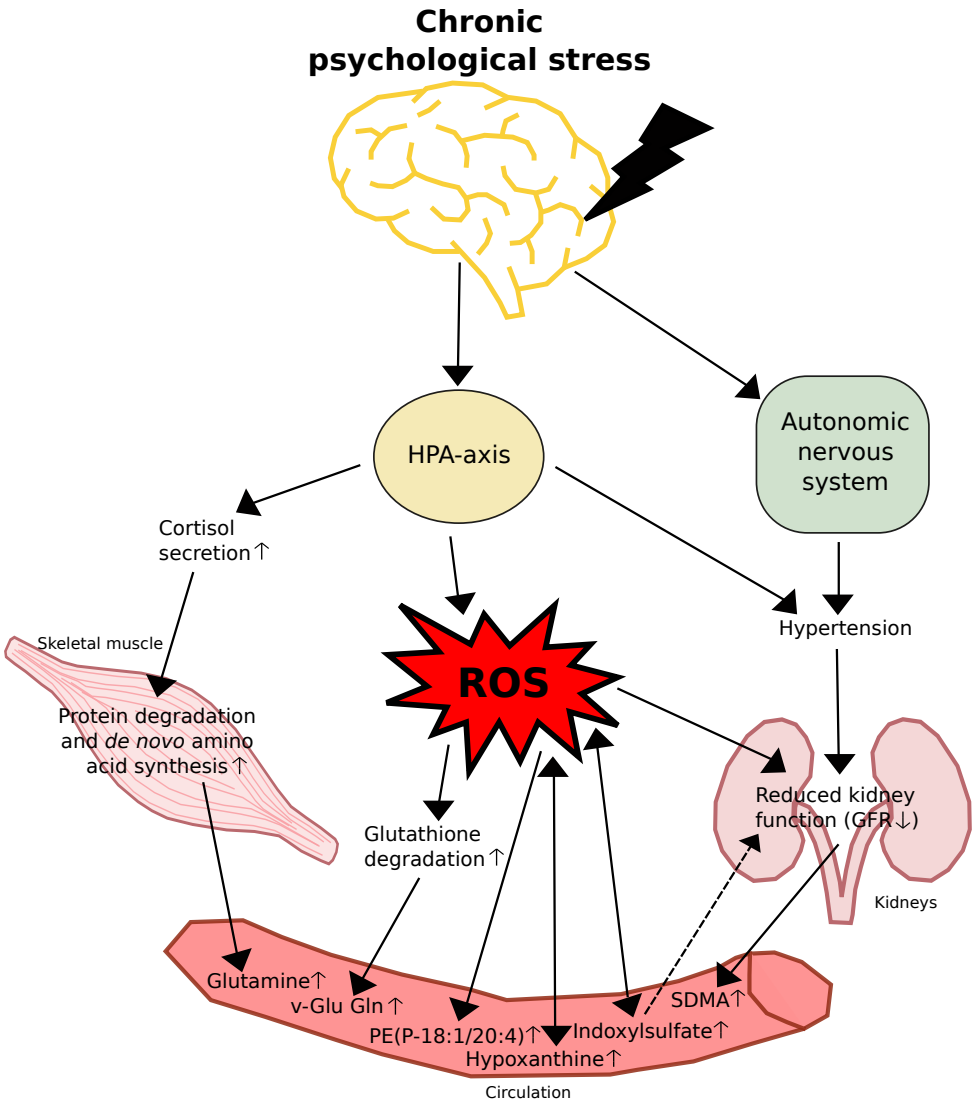

Figure 6. ***A simplified schematic presentation of the possible pathways how the circulating levels of canine fear-related metabolites may be altered by chronic psychological stress.*** *Sustained stress leads to chronic activation of stress response systems, including HPA-axis and autonomic nervous system, with negative health implications, such as elevated cortisol and reactive oxygen species (ROS) levels and hypertension. These events may further alter the circulating levels several metabolites, including glutamine, γ-Glu Gln, PE(P-18:1/20:4), hypoxanthine, indoxylsulfate and SDMA.*

6.2 Both fearfulness and hyperactivity/impulsivity are characterized by decreased phospholipid abundances

In this study, disturbed phospholipid metabolism in both canine fearfulness and hyperactivity/impulsivity was observed. Fearful dogs showed decreased blood phospholipid levels when compared to non-fearful dogs (**Study I**) and hyperactivity/impulsivity was negatively associated with plasma phospholipid abundances (**Study III**).

Phospholipids are molecules where glycerol joins together hydrophobic tail consisting of fatty acids, and hydrophilic head consisting of a phosphate group combined with a characteristic head group, such as choline in PCs (Alberts et al., 2002). They are major components of cell membranes and thus regulate the charge, fluidity and receptor functions of membranes (Conquer et al., 2000; Harayama and Riezman, 2018; Müller et al., 2015). Moreover, they function as signaling molecules, making them important compounds especially in the brain. However, the structural and subsequent chemical diversity among phospholipids is large, and thus different phospholipids may have vastly different biological roles (Harayama and Riezman, 2018).

Lipids have been suggested to have a role in the pathology of psychiatric disorders, and altered phospholipid and fatty acid levels have been associated with anxiety and ADHD (Antalis et al., 2006; Hennebelle et al., 2014; Müller et al., 2015). The lipids and fatty acids seem to be important especially in ADHD, since reduced proportions of polyunsaturated fatty acids (PUFAs) in plasma lipids have been detected in children and adult ADHD patients (Chen et al., 2004; Hawkey and Nigg, 2014; Stevens et al., 1995; Widenhorn-Müller et al., 2014; Young et al., 2004). Especially omega-3 PUFAs, such as docosahexaenoic acid (DHA; 22:6 n-3) and eicosapentaenoic acid (EPA; 20:5 n-3), are decreased in ADHD patients. Omega-3 PUFA deficiency has been suggested to disturb dopaminergic neurotransmission with subsequent attentional and behavioural abnormalities (Delion et al., 1994; Francès et al., 1995; Zimmer et al., 1998). This might explain the link between ADHD and reduced omega-3 PUFA levels.

The results of this study are not directly comparable to findings made in anxiety and ADHD studies in humans and rodents, but suggest that problems in phospholipid metabolism could play a role in canine behavioural abnormalities, too. The problems may lie in the breakdown or synthesis of phospholipids, or in the intestinal absorption of dietary fatty acids and lipids. However, the actual causes and consequences of these findings remain elusive in the context of this study.

6.3 Metabolites associated with hyperactivity/impulsivity suggest altered tryptophan metabolism and gut microbiota

In this study, significant associations were identified between canine hyperactivity/impulsivity and tryptophan metabolites. KYNA showed positive whereas IPA and IAA negative associations with the behavioural variables (**Study III**). These metabolites suggest disturbed tryptophan metabolism in hyperactive/impulsive dogs with possible gut microbiota alterations as they can be or are solely produced by the enteric bacteria.

In mammals, dietary tryptophan can be metabolized via multiple pathways. Majority (~90%) of it is metabolized in the kynurenine pathway under normal circumstances, but serotonin pathway and bacterial degradation have also a role (Keszthelyi et al., 2009) (**Fig. 7**). In the kynurenine pathway, tryptophan is first converted into kynurenine via N-formylkynurenine by the actions of tryptophan-2,3-dioxygenase (TDO) and indoleamine-2,3-dioxygenase (IDO) (Kennedy et al., 2017; Schwarcz et al., 2012). The kynurenine aminotransferases (KATs) are responsible for the conversion of kynurenine further into KYNA. In addition, also enteric bacteria, such as *Escherichia coli*, possessing aspartate aminotransferase (AspAT) activity can synthetize KYNA from kynurenine (Han et al., 2001; Kuc et al., 2008).

KYNA is a neuroactive agent being an antagonist of glutamatergic *N*-methyl-D-aspartate (NMDA) receptor and cholinergic nicotinic acetylcholine α7 receptor (α7nAChR) in the CNS and enteric nervous system (ENS) as well as an agonist of G-protein coupled receptor 35 (GRP35) in the ENS (Kennedy et al., 2017). It has been associated with several CNS disorders, such as schizophrenia and Huntington's disease (Schwarcz et al., 2012). KYNA cannot cross the BBB, and thus is synthetized in the CNS from kynurenine in the astrocytes (Keszthelyi et al., 2009). However, the peripheral KYNA levels are demonstrated to reflect KYNA abundance in the CNS. Moreover, KYNA can influence CNS function also peripherally via the GRP35 function in neuroendocrine cells in the gut.

KYNA is broadly accepted as a potentially neuroprotective metabolite. However, altered KYNA levels in the brain may have also negative implications by disturbing cholinergic, dopaminergic and glutamatergic neurotransmission, all of which are important neurotransmitter systems in ADHD (Schwarcz et al., 2012). Moreover, the positive association between hyperactive/impulsive behaviour and plasma KYNA levels in our study might indicate a shift in tryptophan metabolism favoring kynurenine pathway over serotonin synthesis, leading to serotonin depletion. This hypothesis is highly intriguing as serotonergic dysfunction has been implicated in ADHD pathology in both humans and dogs (Banerjee and Nandagopal, 2015; Wright et al., 2012). However, our results contradict studies in pediatric and adult ADHD patients showing decreased serum KYNA levels in human ADHD (Aarsland et al., 2015; Evangelisti et al., 2017).

The circulating KYNA levels are mainly regulated by the tryptophan levels, the activities of TDO, IDO and KATs, and the gut microbiota. Inflammation as well as psychological and physical stress stimulate tryptophan degradation via kynurenine pathway since proinflammatory cytokines and cortisol stimulate IDO and TDO, respectively (Myint and Kim, 2014). Consistent with this, increased plasma (Dąbrowski et al., 2013) and serum (Dąbrowski et al., 2015) KYNA levels have been previously associated with pyometra, a uterine infection, in dogs.

Besides kynurenine and serotonin pathways, tryptophan can be metabolized also by enteric bacteria (Keszthelyi et al., 2009). This leads to formation of indole and indole acid derivatives, such as IAA and IPA, by bacteria of *Bacteroides, Clostridium* and *Escherichia* genera (Smith and Macfarlane, 1997). In this study inverse associations between hyperactive/impulsive behaviour and plasma IAA and IPA abundances were identified. This suggests differences in the gut microbiota composition between hyperactive/impulsive and non-hyperactive/non-impulsive dogs.

From the gut, IPA is absorbed into the circulation and it can further cross the blood-brain-barrier, having actions also in the CNS (Wikoff et al., 2009; Young et al., 1980). Studies in rodents demonstrate a neuroprotective role for IPA, since it acts as an antioxidant and anti-inflammatory agent (Hwang et al., 2009; Zhang and Davies, 2016). Moreover, IPA has been shown to reduce intestinal permeability (Jennis et al., 2018; Venkatesh et al., 2014). In humans, IPA has been associated with lower low-grade inflammation and decreased risk for type 2 diabetes (de Mello et al., 2017; Tuomainen et al., 2018) and suggested as potential therapeutic option for Alzheimer's disease (Chyan et al., 1999). Thus, adequate IPA production seems essential for health and indicates potential changes in gut microbiotal activity or composition as IPA is solely synthetized by enteric bacteria.

The inverse association between hyperactivity/impulsivity and plasma IPA abundance in our study may indicate several negative consequences for the dogs' health. As intestinal permeability may increase in the absence of adequate amounts of IPA, more deleterious bacterial metabolites can enter circulation having adverse effect on both host's physiological and mental health. This bidirectional signaling between the CNS and ENS, the brain-gut axis, is thought to play a role in the development of psychiatric disorders (Cenit et al., 2017; Groen et al., 2018; Johnson and Foster, 2018). Hyperactive/impulsive dogs may also have compromised antioxidant defense due to reduction in IPA.

Plasma abundance of IAA, a metabolite implicated in ROS production (Dou et al., 2015; de Melo et al., 2004), was negatively associated with inattentive behaviour in our study cohort. However, after controlling for confounding factors, the association was no more significant. This indicates potential age, sex and/or fasting derived effects on the association, and minor emphasis on actual behavioural effects.

Taken together, these findings indicate altered tryptophan metabolism in canine hyperactivity/impulsivity, with potential gut microbiota alterations (**Fig. 7**). The higher KYNA but lower IPA and IAA in hyperactivity/impulsivity suggests overactivation of the kynurenine pathway at the expense of bacterial degradation of tryptophan and with potential reducing effects on serotonin synthesis. Inflammation and stress can stimulate kynurenine pathway by activating IDO and TDO, respectively. This, however, should result also in higher abundance of 3-hydroxyanthranilic acid (3-HK) and quinolinic acid (QUIN), kynurenine metabolites which synthesis is stimulated by inflammation (Myint and Kim, 2014). However, we did not identify these metabolites in our data. The role for altered gut microbiota explaining this difference cannot be ruled out either. Decrease in bacteria responsible for tryptophan degradation in the gut may offer more tryptophan for the kynurenine pathway with subsequent increase in KYNA abundance, but lower levels of IPA and IAA. Moreover, gut microbiota has been demonstrated to contribute to serotonin synthesis, indicating that possible alterations in the enteric bacteria might influence serotonin levels, too (Yano et al., 2015). Nonetheless, additional investigations are required to clarify the causality of our findings.

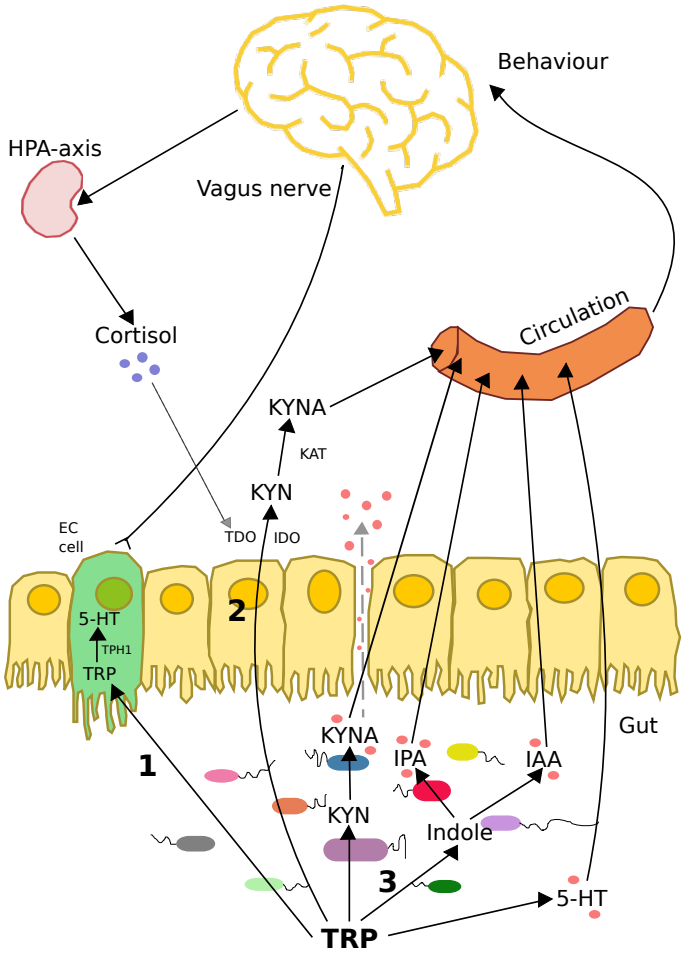

Figure 7. ***Tryptophan metabolism pathways.*** *Dietary tryptophan (TRP) can be metabolized via different pathways, including serotonin pathway (1), kynurenine pathway (2) and bacterial degradation (3). Serotonin (5-HT) can be produced both by gut microbiota and by host metabolism in the enterochromaffin (EC) cells, and it has important functions both in the central and enteric nervous systems. Kynurenine pathway is the major route of TRP degradation where TRP is first synthetized into kynurenine (KYN) which further gives rise to other metabolites, such as kynurenic acid (KYNA). The activity of kynurenine pathways can be influenced by several factors, such as stress via increased cortisol production. Certain enteric bacteria can degrade TRP into several metabolites, including indole derived metabolites 3-indolepropionic acid (IPA) and indoleacetic acid (IAA). The TRP metabolites can influence CNS function with subsequent behavioural changes, making the balance between TRP degradation pathways essential. This balance may be compromised by alterations in the gut microbiota. IDO, indoleamine-2,3-dioxygenase; KAT, kynurenine aminotransferase; TDO, tryptophan-2,3-dioxygenase*

7 Concluding remarks

Existing literature in canine metabolomics is poor. By pioneering the use of a non-targeted metabolomics approach in canine behavioural research, this thesis provides valuable and novel molecular insights into two canine behavioural disorders, fearfulness and hyperactivity/impulsivity. Behavioural problems in dogs and psychiatric disorders in humans represent the most common health issues in both species, with several negative implications for both, the affected itself and for the whole community. As domestic dogs spontaneously manifest similar behavioural and mental health problems as humans, they offer a valuable animal model for human psychiatric research. This is highlighted also by this study, since novel trait-associated metabolic alterations identified in dogs might have significance for basic biological research beyond species boundaries.

The metabolic profile of fearful dogs was characterized by alterations prominently originating from the systemic physiological effects of chronic psychological stress. However, as chronic stress can result from or lead to behavioural problems, the causality of these findings remains unclear. Nevertheless, this study demonstrates the fact that behavioural problems are the major welfare issues in dogs, not only having negative effects on the mental health, but also having a potential to affect the physiological health of the dog.

Hyperactive/impulsive behaviour was significantly associated with enteric bacteria derived tryptophan metabolites, suggesting disturbed tryptophan metabolism with potential gut microbiota alterations. The importance of diverse gut microbiota for mental health has recently been recognized in humans and rodents, and our results suggest that similar effects can be assumed in dogs, too. However, as the connection between the brain and the gut is bidirectional, the changes in tryptophan metabolism and gut microbiota might be both a result of or a cause for behavioural changes.

Decreased circulating phospholipids were linked with both fearfulness and hyperactivity/impulsivity. As phospholipids have major functions as membrane components and signaling molecules, their deficiency might be deleterious especially in the brain. Therefore, they may play an important role in canine behavioural abnormalities too.

We have identified several trait-specific metabolic alterations in dogs in this study. However, much research is still needed to elucidate the actual molecular roles of these metabolites in the pathophysiology of canine behavioural traits before considering them as biomarkers for diagnostic purposes, for example. If validated in repeated and in larger study cohorts, biomarkers could then be utilized as objective and reliable phenotyping tools for research purposes. This might also aid selection of study cohorts which could enhance the potential to major breakthroughs. As metabolism is highly affected by the diet, nutritional interventions to study the possible effects of phospholipids on canine

behaviour, for example, would be beneficial. Ultimately, the identified metabolites can also provide novel targets for drug development.

Conventional single blood chemistry analyses, such as measurement of glucose or inflammation markers have been used for decades in clinical medicine and veterinary medicine for disease diagnostics. However, these methods catch only a minor part of the whole metabolome. More compehensive metabolomics techniques have been intensively developed during the last years. This has made it now possible to cost-efficiently detect tens to hundreds of metabolites in one sample with only one measurement, providing a metabolic fingerprint reflecting global biochemical alterations of an individual. This may have an extreme value especially in the case of complex disorders such as psychiatric and behavioural disorders where global omics-level approaches are needed to obtain a comprehensive view of the disorder etiology and progression. These panels of tens or even hundreds of metabolites have potential to offer huge improvements in health monitoring and disease diagnostics in veterinary medicine in the near future.

Taken together, metabolomics combined with comprehensive clinical and behavioural data, possess massive potential to reveal behavioural disorder-specific metabolic alterations in dogs. These changes may allow insights into underlying pathophysiology and help to gain better understanding of disorder mechanisms. This enables the development of more efficient diagnostic and treatment methods to improve the health and quality of life of our canine companions. Moreover, these findings can benefit human psychiatric research, too. By combining the power and efforts of dog owners, breed clubs and researches, we have all the potential to aid the mental health of both, the man and the man's best friend.

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Helsinki, November 2018

Jenni

Joka liitos natisee
Käy höyry korvista
On aika laittaa lauseelle piste
Uusi virke alkakoon
Se soikoon uljaana
Ihmiskunnan uusi alkukaste

Päämääräkö sen määrää
Missä määrässä
Määränpäässä joku päätäni jo määrää

Tehkää tietä sillä olen irti
Enkä väistä enää ketään
Olen päässyt eroon kahleistani
Enkä pelkää enää mitään

- Irti (M. Annala)

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